NN08201, 2505.

Stellingen bij het proefschrift
van B.M. Schober
" A study on bacterial softrot in witloof chicory",
Wageningen, 20 october 1998.

1. The best adapted subspecies of *Erwinia carotovora* for surviving on the crop, regardless of the source from which it came, will become the predominant strain in the growing season.
(Stanghellini, M.E. *et al.*, Phytopathology 67(1977) 1178-1182)

   Dit verklaart het ruimtelijk gescheiden voorkomen van *Eca* and *Ecc* als veroorzakers van natrot in wilof.
(Dit proefschrift)

2. De rol van koude-tolerante *Pseudomonas marginalis* populaties tijdens de bewaring van witlofpennen wijst op een synergistische verhouding met *Erwinia carotovora*.
(Dit proefschrift)

3. De kennis van de moleculair-biologische achtergronden van de pathogenese van *Erwinia carotovorora* heeft geen invloed op het epidemiologisch onderzoek aan natrot.
(Dit proefschrift)

4. De fysiologische toestand van het gewas witlof en de omgevingsfactoren tijdens de bewaring en trek zijn belangrijker voor de ontwikkeling van natrot dan het voorkomen van de ziekteverwekkers zelf.
(Dit proefschrift)

5. Het belang van de penproduktie voor het voorkomen van natrot in de trek van witlof maakt duidelijk dat ziekten en plagen vanuit het gewas en niet vanuit het pathogeen onderzocht moeten worden.
(Dit proefschrift)

6. Het voorkomen van natrot in witlof is de gezamenlijke verantwoordelijkheid van pennentelers en trekkers.
(Dit proefschrift)

7. ... they (bacteria) have nothing to do with the falsely so-called rotting processes of (dead) plant tissue.
(Hartig, R. 1882. Lehrbuch der Baumkrankheiten. Vertaald door: E.F. Smith. Julius Springer Verlag, Berlin. 198 pp.)

   ...Consequently, there is not the least danger of wound-infectious bacteria, whose further progress in the plant is also impossible.
(Fischer, A. 1897. Vorlesungen über Bakterien. Gustav Fischer, Jena. p.131-132.)

   Conclusie: Bacterieel natrot in witlof is een imaginaire ziekte.

8. Het uitvoeren van praktijkgericht onderzoek door promovendi aan een universiteit genereert niet automatisch in de praktijk toepasbare resultaten.
(zie ook: Wagenings Universiteitsblad, januari 1997).

9. Wetenschappelijke monocultuur, veroorzaakt door grootschalige fusies, leidt tot een verschraling van de "biodiversiteit" onder landbouwwetenschappers.
(zie ook: Vet, L.E.M. 1998. Visie op variatie. Inaugurele rede d.d. 4 juni 1998 aan de Landbouwuniversiteit Wageningen)

10. Kennis is macht.
Dit geldt niet voor het wetenschappelijke onderzoek in Nederland maar voor de financiers ervan.
(zie ook: NRC, 1 september 1997)

11. Men occasionally stumble over the truth, but most of them pick themselves up and hurry off as if nothing ever happened.
Winston Churchill (1874-1965)

12. Hypothesen sind Gerüste, die man vor dem Gebäude aufführt, und die man abträgt, wenn das Gebäude fertig ist. Sie sind dem Arbeiter unentbehrlich; er muß nur das Gerüst nicht für das Gebäude ansehen.
(Johann Wolfgang v. Goethe. Zur Farbenlehre. Tuebingen, Cotta, 1810.)

A STUDY ON BACTERIAL SOFTROT IN WITLOOF CHICORY

Promotor: dr. J.C. Zadoks

Emeritus hoogleraar in de ecologische fytopathologie

B.M. Schober

A study on bacterial softrot in witloof chicory

Proefschrift
ter verkrijging van de graad van doctor
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in het openbaar te verdedigen
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Author's abstract

The production process of witloof chicory, which consists of the root production stage, the storage of the roots and the forcing of the chicory heads, was monitored for the presence of softrot bacteria. In the Netherlands, bacterial softrot in chicory is caused by *Erwinia carotovora* subsp. *carotovora* (*Ecc*) and *Pseudomonas marginalis*. Populations of these species colonised the chicory leaves during the root production stage causing softrot only in the forcing stage. The colonisation incidence, the number of populations of both pathogens present on the chicory leaves, increased during the root production. *Ecc* was inhibited by high radiation levels during the first months of the growing season of the plants. The colonisation incidence of the chicory leaves was influenced by the average temperature throughout the growing season but not by nitrogen fertilisation of the crop or rainfall. The colonisation incidence of the stored roots decreased from harvest time in September until December but increased thereafter. High colonisation incidences during storage coincided with high disease incidence during the subsequent forcing period of the roots. Bacterial softrot occurred during forcing of the roots in all three forcing seasons. Nitrogen fertilisation of the plants, root immersion with calciumchloride and the sampling year had significant effects on disease incidence. Aggregation of diseased chicory heads in forcing trays could be described by an aggregation parameter of the beta-binomial distribution. Studies on the enzyme activity of the softrot bacteria showed that nitrogen and calcium effects on disease severity could be explained by the influence of these treatments on the activity of two pectolytic enzymes of the bacteria. Reduction of bacterial softrot is possible by reduced nitrogen fertilisation during root production and by application of calciumchloride to harvested roots.

The research described in this thesis was funded by the Commodity Board for Agriculture, The Netherlands.

-and you've no idea how confusing it is all the things being alive;

"Contrariwise", replied Tweedledee, "If it was so, it might be; and if it were so, it would be; but as it isn't, it ain't. That's logic."

Alice in Wonderland (Lewis Carroll)

Voorwoord

Vier jaar en zes maanden werk. Vier jaar van lab, veld, lab, bureau, computers. Vier jaar van schrijven, rekenen, meten, discussiëren, leren. Maar dat mag nog, als AIO. En daarnaast grappen maken, mensen leren kennen, computerproblemen verhelpen, meedenken met andermans experimenten. Kortom, vier jaar van mijn leven en daarover nu een voorwoord schrijven.

Mijn allereerste dankwoord gaat dan ook uit aan Theo Ruissen. Hij heeft het project bedacht en geschreven. Hij heeft de eerste aanzet gegeven voor al mijn werk. Zijn manier van denken, vooral over bacteriën, heeft mijn wetenschappelijke visie beïnvloed. Bacterioloog zijn is geen beroep, het is een levenshouding. Als Theo het project heeft opgezet, mijn begeleidings-comissie heeft het bewaakt. Marcel Stallen, Jaap Janse, Gijs van Kruistum, Jim van Vuurde en Jan Carel Zadoks bekeken mijn vorderingen kritisch en hielden mij op het rechte pad van het toegepaste onderzoek. De bijdragen van Gijs en Jim oversteeg de rol van commissielid ver.

Gijs, zonder jouw ervaring van de witlofteelt zou dit boekje ondenkbaar zijn. Jij hebt me wegwijs gemaakt in de witlofproduktie, hebt me voorgesteld aan witloftelers en voor mij zo het probleem waaraan ik werkte levensecht gemaakt. Met jouw hulp en met de hulp van alle mensen van het proefbedrijf van het PAV hebben we vier jaar lang jaarlijks meer dan 200,000 witlofplanten opgekweekt van zaadje tot pen, en daarna tot witlofkrop. Pierre, Joop en Herman: met jullie was de witlofoogst een regelmatig terugkomend feest! En dat ik begon te juichen als een paar honderd kilo witlof stonden te rotten, hebben jullie mij hopelijk al vergeven. Jim, ik heb altijd genoten van onze discussies. Voor jouw zijn bacteriën niet gewoon ziekteverwekkers maar hoogst interessante organismen vol van wonder en schoonheid. Ik hoop dat onze samenwerking een voorbode is van het WURC.

Geen proefschrift zonder praktisch werk. En als men jaarlijks vele duizenden witlofkroppen in alle stadia wil onderzoeken, gaat dat niet alleen. Daarom, op deze ongebruikelijke plaats, dank aan mijn man. Hij is vier jaar lang met mij het veld ingegaan, heeft monsters genomen en helpen verwerken.

Nina en Bert, wij hebben niet lang kunnen samenwerken, en toch is jullie bijdrage aan mijn onderzoek zeer waardevol geweest.

Vele mensen op Fytopathologie hebben mij geholpen, met goede raad of goede daad. Paul Vossen, Richard Laugé en Theo van der Lee dank ik voor hun geduld met mijn naïeve vragen over genen, eiwitten en enzymen. Jos Raijmakers en Mike Jeger hebben altijd tijd gevonden om mijn manuscripten door te nemen en mijn vele vragen te beantwoorden. Dit kan niet altijd makkelijk geweest zijn.
Wetenschap is niet alleen een proces van experimenten uitvoeren maar ook van visies ontwikkelen over de toekomst. Dit heb ik tijdens mijn bestuursperiode in de C.T. de Wit onderzoeksschool Productie Ecologie mogen leren. Het heeft mijn horizon niet alleen verbreed, maar ook "verdiept". Onderzoek opzetten, uitvoeren en van daaruit verder denken. Dit proces was niet mogelijk geweest zonder de bijdragen van Maarten Zwankhuizen, Corné Kocks en Harold Zondag. En over dit heen, als procesbewaking, de sturing van prof. Zadoks. Ik ben hem veel dank verschuldigd voor zijn geduld en de vruchtbare discussies op zijn werkkamer. Ik ben blij dat hij als mijn promotor op wil treden.

"I was born not knowing
and have only had a little time to change that here and there"
Richard Feynman

Contents

Chapter 1

General Introduction

General introduction

Witloof chicory can develop a slimy bacterial rot during forcing and in the post harvest stage. The occurrence of this bacterial softrot and several external factors important for disease development are the topics of this thesis. This introduction provides information about witloof chicory production, the causal organisms of the disease and an outline of the study.

Witloof chicory

The crop
Cichorium intybus L. var. foliosum *Hegi*
Family: Asteraceae (Compositae)

In English speaking countries, the terms "chicory" and "endive" are frequently interchanged because the forced product of witloof chicory has been erroneously named French or Belgian endive. Other synonyms are White Endive, Dutch chicory, common chicory or blue-sailor's succory. Another type of chicory, whose dried roots are used as a coffee substitute, is called Magdeburgh or Italian Dandelion (Anonymous, 1997). Chicory, *Cichorium intybus* L., is a perennial herb native to Europe, North Africa, and Western Asia, and naturalised in North America.
The cultivated plant reaches a height of 1 to 2 meters and has bright blue flowers. Many distinct cultivars of chicory exist, developed by breeding programs designed to meet the different commercial uses of the plant. Centres of chicory production are located in Belgium, The Netherlands, France, and the United States (Van Kruistum, 1997). The reported life zone of chicory is 6 to 27 degrees centigrade with 0.3 to 4 meters of annual precipitation and a soil pH of 4.5 to 8.3. The plant grows best in temperate weather on calcareous soils. Cultivated witloof chicory is sown in spring and harvested in autumn. The harvested material is stored until winter and then placed in an environment conducive to "forcing" and growth in the off-season. An etiolated creamy-coloured head develops in 3 to 4 weeks (Schoofs & De Langhe, 1988).
Chicory is a source of the natural taste modifier maltol, known to intensify the flavour of sugar. The crop is also a potential source of fructose for the flavour industry. The fresh roots contain large amounts of inulin, vitamins A and C, chicoric acid, lactucin, lactupicrin, cichoriin, and several other bitter compounds acting as plant defence mechanism against foraging insects. The phytoalexin cichoralexin is known for its fungistatic. and bacteriostatic properties (Van Genderen & Schoonhoven, 1996; Schoofs & De Langhe, 1988)). Cultivars of chicory developed for use as coffee substitutes have large, thickened roots that are externally yellow and internally white. The roots of these plants are dried, chopped, roasted, and ground for addition to coffee, imparting a strong, bitter flavour. Cultivars of chicory developed for use in salads have more and larger leaves than other cultivars. Chicory extracts are used in alcoholic and non-alcoholic beverages. Young and tender roots can be boiled and eaten as a vegetable. Recently, chicory root is being considered as a natural source of fructose oligosaccharide, a zero-calories sweetener.

As a medicinal plant, chicory root has been used as a digestive aid, diuretic, laxative, tonic, and mild sedative. The root has also been used against jaundice, inflammations, warts, tumours, and cancer. Chicory was thought to purify the liver and spleen. Extracts from the roots have been shown to affect heart tissue isolated from toad (Anonymous, 1997).

Some historical data

The origin of chicory lies in Belgium, where an earlier form has been reported in late medieval times, Barbe de Capucin. This crop consisted of many small growing buds attached to the root. It probably was a cultivated form of *Cichorium intybus* L. var. *sylvestre,* a plant native to Europe, Siberia and Northern Africa (Anonymous, 1997). Egyptians, Greeks and Romans used the green leaves for salads and as a medicinal plant (Schoofs & De Langhe, 1988).
Chicory was grown as a coffee surrogate from the end of the sixteenth century, but not until the nineteenth century were growers able to produce chicory heads as known today. Roots were placed in dark cellars and the heads grew in two to four months time. Technical improvements were made to increase production (De Baeremaeker, 1993). Covering the roots with soil or horse manure accelerated growth, as from then known as forcing. Soil heating with small fires, steam and later with central heating improved quality and increased the production.
Soilless culture of the crop was first developed in the 1950s in Belgium and became common in the 1970s. In the Netherlands, Witloof chicory was first put up for auction in 1913 and because of increasing demand, Dutch farmers started to culture themselves. Around 1930 it was a well established crop in the Netherlands (De Baeremaeker, 1993). Now, after 20 years of further fine-tuning, it is possible to produce chicory year round with a production time of three weeks (Van Kruistum, 1997).

How to produce witloof chicory

Growing the roots
Witloof is sown from mid April until end of May at a depth of 0.5-1cm according to soil and moisture conditions. With graded and pelleted seed, 350,000 - 450,00 seeds are used per ha. After emergence, the plants are thinned to 15 cm spacing in the row with rows 50 or 75cm apart, which results in 200,000 - 250,000 plants per ha. Uniformity of spacing is important since it influences the size of the chicory heads, the chicons, produced.
Plants are fertilised with nitrogen, potassium and phosphorus. Nitrogen fertilisation is crucial, as it determines the quality of the roots as well as the susceptibility to diseases. Low nitrogen content of the soil at the beginning of the growth period is desirable, soils with more than 70kg mineral nitrogen per ha are not suited for chicory production.
Soils with less than 40 kg mineral nitrogen per ha may receive a top dressing of 30kg N per ha before August 15. Witloof performs best under cool temperatures and requires 110 to 130 frost-free days in order to produce roots of desirable size for forcing. Witloof chicory is harvested from the field after 130-150 days, when roots are of adequate size. Roots may be harvested from September through November. Roots should be 3.5-5cm in diameter at harvest and only sparsely branched.

Storing the roots

Roots are harvested by lifting and cutting the root tops 2.5 - 5cm above the shoulders of the roots without injuring the growing point. Failure to remove enough of the tops can result in decay during forcing. Tops may be mechanically removed just prior to undercutting and lifting if this can be done without damage to the growing point. Roots are sorted by diameter and stored in wooden bins or in bulk for pre-cooling and vernalisation.

Vernalisation temperatures prior to forcing affect head quality (sugars and shape), with longer vernalisation producing longer heads. Short-time storage for no longer than few weeks, is done at 3-4°C, long-time storage at – 0.5 - 0°C. To prevent moisture loss, roots are covered with plastic foil and moistured when necessary. Water losses higher than 4% lead to production losses during forcing. Roots commonly are stored in bulk in storage halls or in storage bins of $2m^3$.

Forcing the roots

Roots are cut to a length of 15-25cm and packed tightly and upright in trays 30 cm deep with a hydroponics solution. Both circulating and non-circulating hydroponics systems are used. Roots are forced in darkness in forcing cells. An air temperature of 18-22°C is used for forcing. Ideally, air temperature should be 4-5 °C cooler than water temperature during forcing. Forcing cells are designed with a hydroponics system through which temperature controlled water is circulated. Air temperature is maintained using equipment to control temperature and humidity. Forcing trays are stacked, allowing the necessary space between trays for the growing heads and air circulation. Forcing rooms should be kept at 90% or more relative humidity. Using ideal environmental conditions, 3-4 weeks are necessary for complete head development.

Heads are cut or snapped from the roots and any loose outer leaves are removed. Heads should be 10-15cm in length, compact and spindle-shaped. Heads should weigh 150-300g each and be completely etiolated (free of green colour). They must be handled carefully to avoid wounding and mechanical damage. Hundred kg of roots should yield about 40-50 kg of witloof heads.

Marketing

For transport and marketing, harvested heads are placed in small trays or polystyrene plates with additional shielding of paper or cardboard. They should be shielded from light with paraffin-coated dark coloured paper or plastic as they become green and bitter within hours after being exposed to light. In the Netherlands, approximately 30% of the production is under contract, the rest is sold by auction (Van Kruistum, 1997).

Importance of witloof chicory as a crop in the Netherlands

Chicory is an important field-grown vegetables in the Netherlands. The important root production areas are North-Holland in the north-west, Flevoland in the centre and West-Brabant, Zealand isles and Zealand Flandres in the south-west of the country.

In 1996, an area of 4,000 ha was used for chicory root production in the Netherlands, compared to 14,000 ha in France, 7,000 ha in Belgium and 600 ha in Spain. The Netherlands are the third largest chicory producer in the world. Other countries do force chicory, but import the roots from the major producers.

Total chicory production has decreased in the Netherlands from 90,000 tons in 1991 to 82,000 tons in 1997 due to low prices and sharp competition from France and Belgium. Approximately 30% of the crop is exported, mainly to Germany and Belgium. Prices vary with the season and currently range from fl 3.50 per kg in summer to fl 1.00 per kg in the winter months. The total turnover of chicory at the Dutch auctions was fl. 131,154,000.- in the season of 1996/97 (CBS, 1997).

Important pests and diseases

Pests and diseases occur in all production stages of the crop. Most of the diseases start as (latent) infections during the root production in the field although symptoms may not occur earlier than the late stages of forcing or even in the post-harvest stage. Proper rotations, field selection, sanitation, plant spacing, and fertiliser and irrigation management can reduce the risk of introducing pests and diseases organisms into the crop. Fields free from perennial weeds, where related crops have not been grown for the previous three years, should be chosen to minimise problems with diseases and weeds.

The most important pests during root production in the field are larvae of *Agrotis* species and caterpillars of *Autographa gamma*, both resulting in feeding damage. *Napomyza cichorii* lives in the leaves of the crop and can reach the forcing stage, where it damages the chicory heads. Fruitflies (*Drosphilidae*) can be introduced into storage and forcing as larvae or pupae in the leaf stubs of the harvested roots, during forcing they can transmit softrot bacteria and be a nuisance for people (Van Kruistum, 1987; Boers, 1997).

Nematodes may cause wilting of the plants and malformations of the roots, causing losses up to 40% and resulting in excessively branched roots not suited for forcing. The root-knot nematode *Meloidogyne hapla* and the free-living *Trichodoridae* are significant pests in the Netherlands.

Fungal diseases are reported from all production stages, *Alternaria dauci, Botrytis cinerea, Sclerotinia sclerotiorum* and *Phoma exigua* being the most important in the field. The latter two fungi can cause rotting and quality loss in the harvested roots in storage and during forcing. A fungus causing major damage during forcing is *Phytophthora cryptogea*. This fungus invades the roots and is transmitted in the processing water during forcing. Infected roots produce a slimy root and stop growing.

Several bacterial species can cause severe losses both in the field and during storage and forcing. *Pseudomonas marginalis* causes leaf necrosis in the field and softrot of the chicory heads during forcing. The softrot species *Erwinia chrysanthemi* and *Erwinia carotovora* cause a slimy softrot respectively of the roots and the chicory heads during forcing. Chicory heads with latent contamination by these bacteria may develop softrot in the post-harvest stage and thus are not suitable for exportation but can be sold on the national market.

Bacterial softrot

Causal organisms

Bacteria which cause soft rot include *Erwinia carotovora* subsp. *carotovora*, *E. carotovora* subsp. *atroseptica*, *E. chrysanthemi*, *Pseudomonas marginalis* and *Clostridium* sp. More than one of these bacteria may be involved in a given disease situation.

Softrot bacteria produce enzymes which destroy the middle-lamella between plant cells resulting in a watery, slimy rot.
Initially, soft-rot lesions appear water soaked, sunken and darker in colour than surrounding healthy tissue. Later a whitish, cloudy liquid may ooze from breaks in the plant tissue. Such lesions often have a foul smell. Any plant tissue with such symptoms should be suspected of having soft rot. Fruits, tubers, fleshy roots, fleshy stems, and leaves are susceptible to soft rot. Woody tissues such as old stems are not susceptible. Succulent tissues within woody stems, such as tobacco stems, are susceptible.
Softrot of witloof chicory is caused by several phytopathogenic bacteria, of which *Erwinia carotovora* subsp. *carotovora* (Jones) Bergey *et al.* 1923 and *Pseudomonas marginalis* (Brown) Stevens 1925 are the most common in the Netherlands and *Erwinia carotovora* subsp. *atroseptica* (van Hall 1902) Dye 1969 is found in France and Belgium. In 1992, a new subspecies of *Erwinia carotovora*, subspecies *odorifera* Gallois *et al.* 1992, was isolated from diseased chicory heads in France (Gallois *et al.*, 1992).

Epidemiology
Environmental conditions that are conducive to softrot development include high humidity, abundant rainfall or irrigation, poor drying conditions and relatively warm ambient temperatures. Specifically, temperatures of 22 to 35°C are best for softrot development. Softrot diseases and leaf necrosis (caused by *Pseudomonas marginalis*) in the field often occur in lower, wetter portions of the field where drying is delayed in the morning because of trees and hedgerows.
Infection of plant tissue occurs when free water is present. Natural openings in the plant such as hydathodes and lenticels or abscission tissues such as the leaf stubs of freshly harvested chicory roots are points of entry for softrot bacteria. Wounds, rough handling of produce causing tissue bruising and insect damage are some of the more common points for softrot bacteria. The initial colonisation of the chicory plants happens during the field season, the root production stage, before the harvest of the roots.

A survey by De Boer (1983) on the presence of *Erwinia carotovora* on leaves of potato crops in the Pemberton Valley (Canada) showed a continuous population build-up on potato leaves at one farm location. The pathogens are introduced into the forcing cells on the roots and leaf stubs (Chapters 2 and 3) and symptoms of bacterial softrot appear at the end of the forcing stage (Saane & Van Kruistum, 1987), when the chicory heads are fully grown and densely packed in the forcing trays. Spread of softrot occurs from one chicory head to another, generally causing foci of disease in the forcing trays. Leaking nutrient solution of the hydroponics system and high relative humidity create a water film on the crop which allows the bacteria to move and to reach other heads.

Worldwide importance
Among vegetables in transit and storage, the two subspecies of *Erwinia carotovora* cause more damage than any other storage disease. Their geographical distribution is world-wide and *Erwinia carotovora* possibly occurs in all areas where plants grow without any pathovars being distinguished so far. *Pseudomonas marginalis* with a more restricted host range, has a world-wide distribution as well;

green salad and many *Allium* and *Brassica* species are the economically most important host plants (Goto, 1994).

Pathogenesis

The ability to invade tissues, the capacity to bypass or overcome host defence mechanisms and the production of extracellular substances which facilitate the invasion and make nutrients available are all required for disease development. Softrot bacteria produce a wide range of pectolytic enzymes which attack the middlelamella of the plant cell walls, resulting in plasmolysis and the release of the cytoplasm. Leaked cell contents provide nutrients for the bacteria and act as signalling substances (Barras *et al,* 1994), which increase enzyme production and accelerate pathogenesis. *Erwinia carotovora* produces pectate lyase, a pectin lyase and a endo-polygalacturonase (Pérombelon & Salmond, 1995). These enzymes attack different forms of pectin; their combined attack can degrade almost all possible forms of pectic plant substances.

Treatment

Prevention of disease is most important since no chemical treatments on witloof chicory are allowed in the Netherlands. To prevent disease, several measures can be taken including cultural practices during root production, storage and forcing of the crop, hygiene at harvest and adjusted handling techniques of the crop.

Cultural practices such as nitrogen fertilisation during chicory root production (Saane et al., 1987) are among the key factors in softrot occurrence during forcing. Roots high in nitrogen produce more leaves during the growing season resulting in higher number of bacterial colonies in the field (Chapter 2). Harvest of chicory roots under wet conditions and length of the leaf stubs after defoliation affect the number of places where the bacteria can survive root storage (Chapter 3). Chicory heads grown from roots with a high nitrogen content can develop up to 50% more softrot than roots with a low nitrogen content (Chapter 4).

Calcium influences the quality of chicory roots and heads in many ways. It has widely been used to control physiological deviations of the chicory heads and it also influences bacterial softrot. The application of calcium chloride using root-immersion after harvest (Jolivet *et al.,* 1988) in a 3% solution can reduce the disease by 30-50% (Chapter 4). Hygienic handling techniques of the crop during harvest, regular disinfection of all forcing equipment and clean storage facilities also reduce disease.

Post-harvest disease commonly occurs in chicory heads contaminated with bacteria from cutting knives or by contact transmission of the bacteria after harvest from diseased to healthy cutting areas. Adjusted handling techniques should be applied during the root production, storage and forcing stages. Stringent environmental control during storage and forcing can reduce disease incidence in chicory (Chapters 3 and 4). Ventilation of the stored roots without excessive air velocity minimises moisture condensation and facilitates quick cooling of the roots, increasing the bacterial decline on the roots. Lower air temperatures and a relative humidity below 95% during forcing can reduce disease severity further.

Outline of this study

History of the problem

The introduction of the hydroponics system for forcing, which reduced forcing time to three weeks, increased disease incidence on chicory heads and altered the composition of the bacterial flora (Vantomme, 1989). Losses of 50% and more of the total production occurred regularly during early forcing in hydroponics systems when susceptible cultivars are used. Since bacterial softrot is caused by bacteria with a wide host range and the ability to adapt to a wide range of environmental conditions, it is difficult to design measures for the reduction of disease incidence and severity. The complexity of the chicory production (see before) and the intensity of the forcing procedure created plants more susceptible for softrot, especially in the early forcing season from September until December.

Objectives

The aim of this study is to gain knowledge on the ecology of softrot bacteria during the three production stages, root production, root storage and forcing of the roots. Chicory plants are colonised during the root production stage in the field. Plant debris, wild plants, neighbouring dicotyledonous crops and the soil form infection sources. During the storage phases and the early forcing period, the number of bacterial colonies increases.
Disease symptoms begin to appear late in the forcing period and at harvest of the chicory heads, sometimes even in the post-harvest stage. Conditions which lead from latent colonisation of the plants to disease development and the factors favouring the development of populations of softrot bacteria were studied in more detail. Detailed information on the ecology and epidemiology of the softrot bacteria create a knowledge basis from which strategies for disease management and treatment can be developed.

Approach

The occurrence of softrot bacteria during the production stages of chicory and the physiological requirements for their permanent establishment on the plants were studied. Latent infections of the crop, which lead to softrot, have specific requirements for nutrients and environmental conditions, otherwise the bacterial populations will become extinct. Starting with data derived from earlier work on bacterial softrot (Saane & Van Kruistum, 1987) several factors which might influence bacterial softrot in chicory were chosen for research.
The main hypothesis underlying the present study was the importance of cultural and environmental factors on disease incidence and severity. Several aspects from three production stages of the chicory production were to be studied in detail. The influence of nitrogen management and of environmental factors such as temperature and radiation during root production were studied for their effect on the occurrence of softrot bacteria in the field as latent infections.
Temperature and relative humidity during root storage studied since they determine the chances for survival of the bacteria on the crop. The application of calcium-chloride to the roots after harvest and forcing conditions are known to regulate disease severity (Van Kruistum, 1997), and thus their effect on softrot incidence was investigated.

Topics studied

A method to quantify softrot bacteria in latent infections of saprophytic colonies during the root production and storage phases was developed to describe the infection process and the population dynamics (Chapter 2). In an attempt to comprehend the problem – as softrot bacteria are omnipresent and current production practice of chicory does not facilitate effective disease management, it was hypothesised that more knowledge of the physiology on the bacteria during early pathogenesis might help to develop new control strategies (Chapter 3).
In addition, the water availability and temperature necessary for the development of softrot bacteria was studied as a possible starting point for disease control during the forcing of the roots (Chapter 4). A description of the problem was undertaken using quantitative methods to determine the colonisation incidence and qualitative methods to determine the causal organisms during the three production stages of chicory, the root production in the field (Chapter 5), the storage of the roots (Chapter 6) and the forcing of the roots (Chapter 7).

Chapter 2

Detection and enumeration of *Erwinia carotovora* subsp. *atroseptica* using spiralplating and immunofluorescence colony staining (IFC)

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B.M.Schober and J.W.L. van Vuurde

Summary

Immunofluorescence colony staining (IFC) and a new technique using spiralplating combined with IFC, were evaluated for the soft-rot pathogen *Erwinia carotovora* subsp. *atroseptica* (*Eca*) in witloof chicory. Target bacteria could be detected in platings at various dilutions of plant washings. Brilliance of the stained colonies of *Eca* was high. Spiralplating, used for both the plating of the bacteria and for the delivery of the conjugated antiserum had a positive effect on the reduction of the background compared to the staining of the bacteria. The combination of spiralplating and IFC proved to be a functional tool for quantification of target and non-target bacteria and the isolation of target bacteria as pure culture from IFC-positive colonies. The method uses less conjugated antiserum than traditional IFC and produces results with very small within replications variation. The recovery of the bacteria in both pure culture and plant washing is significantly higher than the recovery using Crystal Violet Pectate medium.

Introduction

The aetiology of soft-rot in witloof chicory (*Cichorium intybus* L. var. *foliosum* Hegi) is complex and confounded by the uncertainty about the causal organism (Friedman, 1951; Pérombelon & Salmond, 1994). The aetiology may involve several organisms acting alone or in concert. The bacteria only occasionally cause disease during root production (Sellwood & Evart, 1981), but they can survive and cause latent infections which are carried along into storage and forcing. Colonisation of witloof chicory leaves by epiphytic populations of the bacteria *Erwinia carotovora* (Jones) Bergey *et al.* 1923 and by *Pseudomonas marginalis* (Brown) Stevens 1925 has been observed during the root production stage (Vantomme *et al.*, 1989; Van Outryve *et al.*, 1988; Schober, 1998). These bacteria can turn into pathogens under suitable environmental conditions (Liao & Wells, 1987).

Erwinia carotovora subsp. *atroseptica* (*Eca*) has been reported to be the major causal agent of bacterial soft-rot in France and Belgium during forcing of the roots (Samson *et al.*, 1980; Bouvard, 1987). The risk of bacterial rot of chicory heads during forcing and post-harvest treatments is of constant concern to chicory producers, particularly because there is no effective chemical control for bacterial soft-rot. With little prospect of an effective bactericide, cultivation practices are the only reliable measures.

Assessment of the health status of the roots and their contamination with soft-rot bacteria can help chicory producers in decision making. Therefore, reliable techniques for the detection, identification and enumeration of the bacterial populations on the plant itself are needed.

With traditional techniques, the detection level of cultivable target bacteria is often limited to about 10^4cfu/ml soil or plant washing because of the excess background bacteria in the sample (Van Vuurde and Van der Wolf, 1995).

Furthermore, the recommended selective medium designed for the detection of pectolytic *Erwinia* spp., Crystal Violet Pectate (CVP, Cuppels & Kelman, 1974), does not discriminate between the different *Erwinia* subspecies. These subspecies have different properties regarding the aetiology of soft-rot and the severity of the loss, which makes discrimination for assessment purposes crucial.
The detection level of immunofluorescence colony staining (IFC) is often more than 10 times more sensitive than that of selective media and the detection of the target organism is not hampered by the presence of background bacteria (Jones *et al.*, 1994). However, enumeration of high numbers of target colonies in IFC is laborious and the efficiency of re-isolation of target bacteria from IFC-positive colonies may be below 50% in samples with more than 500 colonies per cm^2 (Van Vuurde & Roozen, 1990).
This paper reports on a new technique to quantify and qualify, i.e. detect, the soft-rot bacterium *Eca*. The objective of this research was the development of a combined technique of spiralplating and IFC which offers maximum recovery and qualitative assessment of *Eca* in IFC and of isolation of target colonies from IFC-positive colonies. Spiralplating allows for the plating of a 40-100 fold dilution series in a 2-dimensional distribution of the bacteria onto one agar plate, thus enhancing the recovery of target organisms and increasing the reliability of counting. The IFC technique has a low detection limit (100 cfu/ml) of the target bacterium and combines detection and serological identification into one step. In the combination of a non-selective medium with IFC, the target bacteria can be detected, quantified and related to the total number of bacteria present in the sample.

Material and methods

Bacterial strain

Erwinia carotovora subsp. *atroseptica (Eca)* (no.161) from the culture collection of the IPO-DLO (Research Institute for Plant Protection, the Netherlands) was used and maintained on Nutrient-agar (Oxoid CM3) at 27°C.

Inoculum

Cell suspensions of *Eca* were made from 24-48 h cultures (grown on Tryptic Soy Agar (TSA, Oxoid CM 131) at 27°C) in 0.1 M sterile phosphate buffered saline (PBS) at pH 7.2. Densities were determined with a photospectrometer at 600 nm and a calibration curve, and afterwards adjusted to values of 10^5 cfu/ml or 10^8 cfu/ml using PBS.

Preparation of the plant washings

Experiments were done with chicory heads cv. RUMBA or SALSA that had been forced at the Research Station for Arable Farming and Vegetables (PAGV), the Netherlands. Strips of leaves from chicory heads were washed in 5 ml PBS + 20 ppm Tween 20 using a Vortex shaker at maximum speed.

Washings were centrifuged (10.000 rpm, 5 min), the pellets containing epiphytic micro-organisms, were pooled and resuspended in PBS. Densities were calibrated with PBS at 600 nm to an absorption of 0.500 A (corresponding to a density of ca. $5*10^9$ cfu/ml). Appropriate densities of *Eca* were added to the suspension to obtain a final density of *Eca* of 10^5 cfu/ml and 10^8cfu/ml. This mixture was used for enumeration in spiralplating.

Spiralplating

An Autoplate™ model 3000 from Spiral Biotech was used to deposit a sample of 50 µl in a spiral pattern onto the surface of a rotating agar plate. The resulting growth pattern can be used to assess the density of the bacteria in the sample. Due to the automated process, variation between replicates is minimised and follows a Poisson distribution (Autoplate™ User Guide, 1993).
TSA was used in a 20-fold dilution. Additional agar (Agar no.3, Oxoid) was added to a final concentration of 1%. Successively, 8 ml of melted agar was poured into 90 mm Petri dishes and dried for 30 min in a flow cabinet with the lid off at room temperature. The bacterial suspensions were then plated with the Spiralplater using the exponential mode (according to the manual of the manufacturer) and left to dry for another 30 min. Platings were topped with 3 ml of the same agar at 34°C to limit the growth of the bacterial colonies on the agar surface. Plates were incubated for 24-48 h at 27°C until the colonies of the target bacteria were just visible without magnification (ca. 0.1 mm). After incubation, dishes were washed in 10 ml 0.1M PBS with 20 ppm NaN_3 for 4 h on a rotary shaker to remove soluble antigens and incidental colony growth at the surface of the agar. Total numbers of bacteria were assessed using the counting method of the manufacturer.

Immunofluorescence colony staining

Antiserum
FITC-conjugated antiserum (no. 8898 F-L5/11 with a IgG concentration of 3 µg/ml) was obtained from the IPO-DLO. The purification and conjugation of the polyclonal antiserum were described previously (Van Vuurde & Van der Wolf, 1995). All dilutions of the antiserum were done using 0.01M PBS with the addition of 1 unit of agarase (Sigma) per ml.

Direct staining with the Spiralplater
Washed Petri dishes with visible colony growth were surface air-dried in a flow cabinet for 30 min. The conjugated antiserum was plated with the Spiralplater using uniform-lawn mode, dispensing 20 µl at a constant rate onto each plate (according to the instructions of the manufacturer). Conjugate was used in 10 to 100-fold dilution. Dishes were incubated overnight at 27°C and washed again for 30 min up to 2 hours to remove excess antiserum. After discarding the washing fluids, the plates were examined under the microscope. Addition of 20 ppm NaN_3 to the washing fluid allowed the storage of the plates in the refrigerator for further use.

Staining of the colonies in a 24 wells culture plate
Selected areas showing separate bacterial colonies were cut from the spiralplating dishes with a sterilised cork bore (diameter 7 mm) and were transferred to the wells of a 24-wells tissue culture plate (Costar), two punches per well. To each well 500 µl 0.1M PBS with 20 ppm NaN_3 were added and the plates were washed on a rotary shaker for 4h. Washing liquid was removed with a vacuum pump and 350 µl conjugate (diluted 50- or 100-fold) was added per well; after overnight incubation, the contents of each well were washed in two changes 600 µl PBS per well on a rotary shaker for 30 min.

Examination

All Petri dishes were examined under a Wild binocular, equipped with incident blue light (Van Vuurde & Van der Wolf, 1995) and 5x or 10x oculars at 6-31x final magnification to check overall staining and screen for cross-reacting non-target bacteria. Closer examinations were made with a Leitz Orthoplan using 4x oculars and Leitz 4x/NA 0.12 or 6.3x/NA 0.20 objectives or a Nikon SLWD 10x/NA 0.21 objective with incident blue light from a UV mercury lamp (200 W high pressure), a 450-490 nm excitation filter and a 510 nm barrier filter. Fluorescence intensity of the target colonies was evaluated a scale from 1-5. 1 "unstained", was defined by the fluorescence of non-target colonies, not the agar itself and 5 showing maximum brilliance of target colonies. The background staining was evaluated using the same scale.

Image analysis
A SUN-based image analysis-program, GOP302, was used to assess brilliance of the colonies automatically. A black and white camera (Fujitsu) was used to take pictures of the samples of 6 mm diameter which were directly processed by the image analysis program assessing the grey value of both the colonies and the background. For image analysis, the background was calculated as the average light intensity of both non-target bacteria and uncovered agar.

Evaluation of the assays

Effectiveness of the conjugated antiserum
The highest effective dilution of the antiserum was determined in a dilution series for both spiralplating-IFC and 24-wells IFC assay. Positive controls were agar preparations containing *Eca* used for IFC (Van Vuurde & Roozen, 1990; Van Vuurde & Van der Wolf, 1995) and negative controls were samples without conjugate but with the equivalent amount of buffer and agars. All dilution series were tested with low (ca. 10^5 cfu/ml) and high (ca. 10^8 cfu/ml) densities of *Eca*. Image analysis was used to obtain objective data for brilliance of colony-fluorescence and background staining.

Recovery of *Eca* in the assays

Pure cultures. Dilution series of *Eca* ranging from 10^1 to 10^8 cfu/ml were used for spiralplating on TSA, CVP (20 µl/plate) and 1/20 TSA for IFC. Recovery was determined by spiralplating counts for TSA and by counting pits in the CVP medium.

Chicory microflora. Suspensions of chicory microflora and *Eca* were prepared from 24-48 h cultures (grown on TSA at 27°C) in 0.1 M PBS. The densities of the suspension ranged from 10-10^8 cfu/ml. Recovery was calculated as the percentage of colonies found on IFC or CVP related to the number of colonies in pure culture suspensions on TSA.

Re-isolation. Locations of IFC-stained colonies of *Eca* in chicory microflora were marked using a Wild stereo-microscope and re-isolated by plating them on TSA plates. After incubation at 27°C for 6 days, the purity of the isolates was checked visually at 6x magnification. Re-isolation was performed for *Eca* densities from 10^1 - 10^7 *cfu/ml with a background of* 10^5 *cfu/ml of chicory microflora.*

Results

Evaluation of assays

Spiralplating-IFC and 24-wells IFC-staining showed IFC-positive colonies of *Eca*. Antiserum reacted with the target bacteria until 100x dilution of the conjugate in both techniques, but at lower concentrations better results were obtained with the 24-wells technique (Table 1). Fluorescence brilliance was higher than the staining of the background, on average brilliance was 3 on a scale from 1-5.. For 24-wells IFC, brilliance ranged from 2.8 to 4 and for spiralplating IFC from 2 to 3.5. Lower densities of *Eca* resulted in bigger colonies, which often showed a stained halo of precipitated diffusable antigens around the colonies. The non-target bacterial background staining of the samples had an intensity of 1.
Colony staining differed significantly (Student's t-test, $p < 0.05$) from the background staining for both *Eca* densities and all conjugate concentrations. The ratio of colony-brilliance and background staining (BR/BG ratio) on average ranged from 1.5 to 2.5 (Table 1), optimum ratios were found for high concentrations of the antiserum and low *Eca* densities in the 24-wells IFC. The BR/BG ratio was not influenced by the bacterial densities or the antiserum concentration in the spiralplating IFC. Using high concentrations of *Eca* resulted in very small colonies of both *Eca* and chicory microflora. The brilliance of the staining was unchanged but visibility was hampered by the small size. Using a 10x objective in the microscope solved this problem.

Concentration of the conjugate

For the 24-wells technique, the best concentration was a 150 dilution and for spiralplating-IFC it was 120 dilution. In 24-wells IFC, higher concentrations of the antiserum did not improve the brilliance of the target colonies nor had any influence on the background staining (Table 1). In spiralplating-IFC, results up to a 100-fold dilution of the conjugate were comparable with the 24-wells IFC. Lower densities of the bacteria resulted in greater colony size. The increasing colony diameter resulted in some decrease of the brilliance of the staining, but had no effect on background staining. In general, brilliance of target colonies was higher at 110 and 120 dilutions of the conjugate. For 24-wells IFC, 3µl of undiluted conjugate were needed per well (i.e. 200mm^2 agar) for the optimum staining of the colonies, for spiralplating-IFC staining of a Petri dish (i.e. 6300 mm^2 agar) required 4µl of undiluted conjugate.

Image-Analysing of 24 wells plates

Processing the 24 wells culture plates was possible with an 2.5X objective. Per well, both IFC-stained agar punches were screened, resulting in two images per well. Brilliance of the *Eca* colony staining and background staining were measured at a grey scale ranging from black (0) to white (1000).

Results were pooled and processed by the image analysis program. Background staining decreased with lower concentrations, a 150 dilution of the conjugate gave the best results with the lowest variation of colony staining (Table 2). For all concentrations, brilliance differed significantly (Student's t test, $p < 0.05$) from the background staining. Processing data of 24-wells IFC with high densities of *Eca* was not accurate because the colonies could not be separated by shape recognition of the image analyser. This resulted in a reduced recognition of the colonies. Brilliance of the staining ranged from 60-200 and a background of 3.

Table 1: Comparison of different concentrations of the conjugate in 24-wells IFC and spiralplating-IFC at low (10^4 cfu/ml) and high (10^8 cfu/ml) densities of *Eca* in plant washings. Data are means and standard deviations of 15 replicates (24-wells IFC) and 2 replicates (spiralplating-IFC)

IFC format	Antiserum dilution	Density *Eca*/ml	Colony brilliance[1]	Background fluorescence[2]	BR/BG ratio
24-wells IFC	150	10^4	4.0 ± 0.4	1.5 ± 0.2	2.7 ± 0.45
		10^8	2.8± 0.6	1.7 ± 0.3	1.7 ± 0.48
	1100	10^4	3.5 ± 0.4	1.4 ± 0.4	2.7 ± 0.84
		10^8	2.9 ± 0.4	1.9 ± 0.3	1.6 ± 0.33
Spiralplating-IFC	110	10^4	3.0	1.2	2.5
		10^8	3.5	0.6	5.8
	120	10^4	3.5	1.5	2.3
		10^8	3.5	1.8	1.9
	150	10^4	2.5	1.2	2.1
		10^8	2.5	1.2	2.1
	1100	10^4	2.0	1.2	1.7
		10^8	2.0	1.2	1.7

[1] Colony brilliance (BR) of IFC-stained target colonies is expressed on a scale from 1-5, 1 being very weak and 5 being very brilliant.

[2] Background fluorescence (BG) is measured for non-target bacteria and expressed on a scale from 1-5, 1 being as dark as a non stained sample and 5 being of the same brilliance as colony brilliance 5.

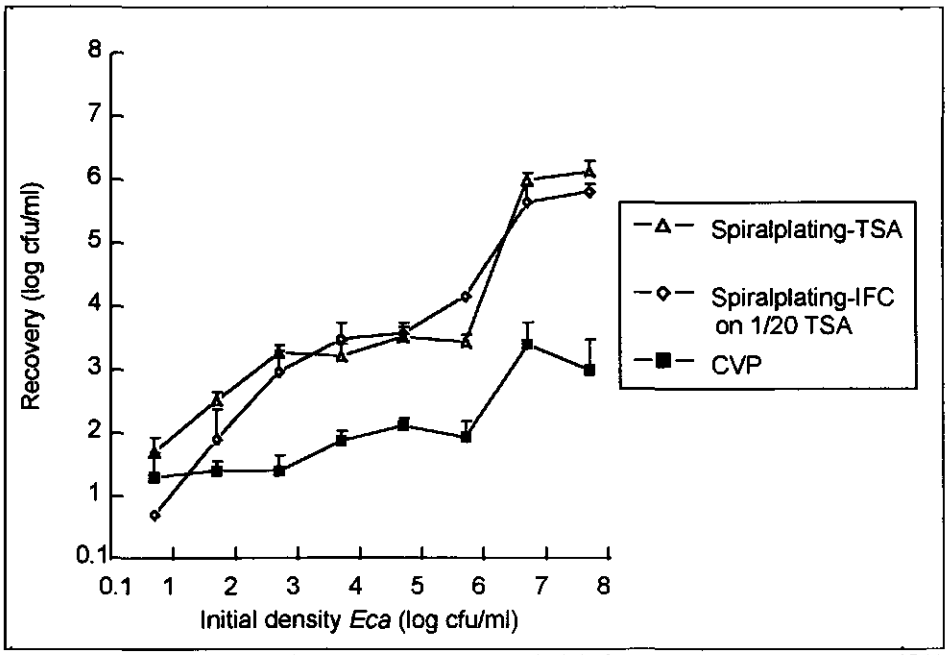

Fig 1 Recovery of *Eca* in plant washings using spiralplating on a general medium (TSA), spiralplating-IFC and a selective medium (CVP). Recovery is calculated using numbers of *Eca* on TSA in pure culture as a reference. Data are means and standard deviations of 8 replicates.

Recovery

Eca in pure culture was recovered equally well with CVP plates and the spiralplating-IFC technique. Variation within replicates was low. Both CVP plate counts and numbers of stained colonies in spiralplating-IFC of *Eca* in chicory microflora were comparable with counts obtained on TSA (Figure 1).

The recovery for the densities of *Eca* from plant washings used in the experiment did not differ significantly ($p > 0.05$) for the techniques used.

Recovery of *Eca* did not reach 100% using spiralplating on TSA of pure cultures of *Eca*. This resulted in an overestimation when calculating the recovery as the number of colonies found with CVP plating or spiralplating-IFC compared to the number of colonies found with spiralplating on TSA. Recovery of *Eca* in plant washings was low for CVP plates, with an average recovery of 10% for low densities of *Eca* and <1% for high densities of *Eca* compared to counts of *Eca* densities on TSA in pure culture. Also, variation within replications was very high on CVP plates (data not shown).

Re-isolation

IFC-positive colonies of *Eca* on spiralplating dishes which were lying separated from other bacterial colonies were used and marked for re-isolation. Pure *Eca* cultures were obtained in the first plating for pure cultures from the re-isolated IFC-positive colonies in 75% of 30 attempts. In the other 25% of all attempts, culture plates showed bacterial growth of both *Eca* and contaminants, and needed additional purification. Density of *Eca* in the spiralplating IFC had a positive effect on the possibilities to obtain a pure culture immediately after re-isolation. Re-isolation was possible for all *Eca* densities, but most effective between 10^1 and 10^5 cfu/ml of *Eca*, due to the larger colony size.

Discussion

Specificity and sensitivity of spiralplating-IFC

The specificity of a serological detection technique is determined by I) the properties of the media, II) the antisera used and III) the observation methods. Specificity expresses the capability of a technique to minimise unspecific reaction with non-target bacteria which produce false positives (Sheppard *et al.*, 1986).
False positives often can be distinguished from target colonies by their size or shape. Furthermore, re-isolation of stained colonies and their identification using standard procedures for the identification of micro-organisms should be performed routinely.
Comparing the numbers of *Eca* colonies in spiralplating on a general medium and the numbers of IFC-positive colonies found in spiralplating IFC, there should be no significant difference between both methods. As shown in figure 3, numbers of bacteria were comparable for TSA plating and spiralplating-IFC. In our experiments, numbers of *Eca* colonies detected from suspensions of a pure culture did not differ significantly from numbers of colonies of *Eca* detected in plant washings of witloof microflora.

Table 2: Image analysis of 24-wells IFC staining using 2.5x objective magnification and grey scale analysis for low densities (10^4 cfu/ml) of *Eca* in plant washing, data are means and standard deviations of 19 replicates

Antiserum concentration	Colony brilliance[1]	Background fluorescence[2]	BR/BG ratio
16.25	150 ± 52	2.5 ± 0.6	62 ± 24
112.5	111 ± 17	3.2 ± 0.8	38 ± 15
150	211 ± 25	3.2 ± 1.5	76 ± 27
1100	126 ± 29	2.0 ± 0.0	63 ± 15

1, 2 see table 1.

Using the selective medium CVP for the detection of *Eca* in witloof microflora reduced the recovery significantly. In image analysis, yellow autofluorescence of non target-bacteria could cause difficulties. Image recognition of the target *Eca* colonies requires some tolerance in defining shape and size parameters, resulting eventually in confounding with non target bacteria.

The sensitivity of a detection technique is defined by the risk for false negatives (Sheppard *et al.*, 1986). For IFC-detection this means that no unstained target colonies should occur after the reaction with the conjugated antiserum. Standard 24-wells IFC as well as spiralplating-IFC depend on the quality and specificity of the antiserum used. Furthermore, the conditions during the experiment should promote the uniform growth and development of the colonies to minimise variation in the binding of the conjugated antiserum.
In our experiments, the staining of the target colonies was slightly negatively correlated with the diameter of the colony and the production of soluble antigens by the colony itself which dissolved in the agar and resulted in a stained halo around the colony. Growth of the colonies as well as reduction of soluble antigens could be optimised by modifying the composition of the agar and minimising incubation periods after spiralplating of the bacterial suspensions. The recommended dilutions of 120 of the conjugate for IFC-staining of plates gave reliable results in our experiments

Evaluation of the assay

The combination of spiralplating and IFC proved to be a functional tool to detect and quantify *Eca* in plant washings. Spiralplating IFC uses less conjugate than standard IFC techniques and produces results with little within replications variation. Recovery of the bacteria both in pure culture and in plant washing is generally high and comparable to standard pourplating IFC.
Own results as well as earlier work showed that recovery was higher in IFC than in detection using CVP plates (Van Vuurde & Roozen, 1990; Jones *et al.*, 1994). CVP is used for the detection of pectolytic bacteria of which *Erwinia carotovora* ssp. produce deep small pits in the gel after 24-48 h. Other pectolytic bacteria produce shallower pits. In our experiments recovery on CVP was poor, due to microbial antagonism on the plates as found by Jones (1994) and the toxic properties of the pectate used (Pérombelon & Burnett, 1991). In particular, high densities of chicory microflora from the plant washings resulted in very low numbers of cavity forming colonies of *Eca*. Furthermore, cavity formation can not be attributed to *Eca* alone, other pectinolytic *Erwinia's. Pseudomonas* and *Bacillus* species form cavities on CVP (Pérombelon & Burnett, 1991).
In particular, colonies of *Erwinia carotovora* subsp. *carotovora* can not be distinguished from the target colonies of *Eca*. Jones *et al.* (1994) showed that selection of pectinolytic *Erwinia* spp. by temperature differentiation is not effective for their detection in plant washings. Own results show a recovery of ca. 50% of *Eca* using spiralplating IFC in chicory microflora compared to counts of *Eca* in pure culture. Using the standard 50 µl per sample in spiralplating, as little as 40-100 cfu/ml were detectable.
The detection limit of 10 -100 cfu/ml of traditional IFC is obtainable, but observation time is increased, especially at low *Eca* densities. One major reason of a hampered detection of *Eca* is microbial antagonism or interaction which can obstruct the growth of the bacteria.

Due to the dilution effect of the spiralplating process and the restriction of biomass per colony because of the top agar layer, these effects have not been observed throughout these experiments. Due to the two-dimensional spread, target colonies can be easily re-isolated for confirmation by further characterisation or other purposes. Re-isolation of *Eca* colonies was satisfactory and purity of first platings was obtained in 75% of all attempts. In our opinion, spiralplating IFC can be a useful tool for ecological studies evolving pathosystems where suitable polyclonal or monoclonal antibodies are readily available for quantification and isolation of the target bacteria.

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Chapter 3

Enzymatic maceration of witloof chicory by the softrot bacteria *Erwinia carotovora* subsp. *carotovora*

Enzymatic maceration of witloof chicory by the softrot bacteria *Erwinia carotovora* subsp. *carotovora*

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Summary

Disease incidence of bacterial softrot caused by *Erwinia carotovora* subsp. *carotovora (Ecc)* and activities of bacterial pectolytic enzymes were studied in witloof chicory. Disease incidence after forcing of the chicory heads depended on the nitrogen and calcium treatment of the chicory plants prior to forcing. Nitrogen, calcium and the forcing season had a significant effect on disease incidence in cultivar Rumba. Significant differences in susceptibility to bacterial softrot were found for the tested chicory cultivars. Disease severity after 96 hours ranged from 6% in Clause R2 to 100% in Bea and Tabor. Chicory cultivars Rumba and Salsa showed a final average severity of 65 - 70%. Activity of the pectolytic enzymes polygalacturonase and pectate lyase increased in artificially inoculated chicory heads of cultivar Rumba, polygalacturonase showed highest activities 48 hours after inoculation and pectate lyase 72 hours after inoculation. Maceration of the chicory tissue and bacterial growth increased continuously until 96 hours after inoculation, when more than 60% of the chicory heads was macerated by pectolytic enzymes of the bacteria. Enzyme activity of *Ecc* grown on cell wall extracts from chicory cultivar Rumba was not influenced by the nitrogen and calcium treatment of the chicory plants. The activity of polygalacturonase reached its highest levels 48 hours after incubation and pectate lyase activity decreased continuously until 72 hours after inoculation. Growth of *Ecc* was not affected by the calcium or nitrogen treatment.

Introduction

Bacterial soft rot is characterised by maceration and cell rupture in fleshy parts of a plant. Symptoms are caused by disintegration of plant tissue by softrot bacteria among which *Erwinia carotovora* subsp. *carotovora.* Pectic enzymes are released into the tissue by soft rot bacteria causing the disintegration (Collmer, 1987; Lei, 1985). The extracellular pectic enzymes are the primary factor causing rot. Pectic enzymes initiate the maceration of plant tissue by digestion of the cell walls and the middle lamella of the cells. Subsequent cell rupture makes cellular fluids accessible for the bacteria, causing bacterial growth which results in increasing enzymatic degradation of the tissue. The first enzymes which are produced by the bacteria do not require external stimuli for production or activation, they are produced constitutively (Tsuyumu & Chatterjee, 1984).

Digestion products of pectate stimulate the production of other pectic enzymes in great amounts. The enzymes then cause massive maceration of the tissue, resulting in an increase of the bacterial population and further enzyme production. The first symptoms of bacterial softrot are browning of the tissue and visible maceration of the diseased plant parts.

Earlier studies (Knösel, 1967; Pagel, 1994) have indicated that the virulence of softrot bacteria is closely linked to the production of pectolytic enzymes.

Their production depends on the environmental conditions and the host plant involved (Zucker, 1972). Several factors affect tissue maceration, especially calcium ions and the pH of the plant tissue (Pagel & Heitefuss, 1990).

Calcium ions have opposite effects on the activity of the two major enzymes during early pathogenesis, calcium stimulates pectate lyase activity and inhibits polygalacturonase activity (Alghisi & Favaron, 1995). The nitrogen content and the pH of the plant tissue affect the pectolytic enzymes in more than one way. The presented study focused on the effects of the nitrogen content of chicory roots and the application of calciumchloride as a root immersion treatment on the amount of maceration caused by softrot bacteria. Nitrogen and calcium contents of the crop were varied by standard cultural practices during the witloof chicory production. The effects of nitrogen and calcium status of the crop on the enzyme activity of the bacteria were studied. We focused our study on the consequences of these influences on softrot development during forcing of the chicory heads of several chicory cultivars. Moreover we compared the activity of the pectolytic enzymes in inoculated chicory heads and in cell wall extracts from chicory heads as a pectate source.

Material and methods

Plant material

The crop was produced following standard practices at the Experimental Station for Arable Farming and Vegetables as described previously (Schober & Zadoks, 1998). Witloof chicory cultivars Salsa , Rumba, Tabor, Bea and Clause R2 were used in the experiments. Nitrogen fertilisation during the root production stage and a calcium treatment of the roots after harvest were applied. Nitrogen fertilisation (indicated as high nitrogen) was applied during the root production stage in two applications, one as a pre-sowing application of 70 kg ha^{-1} and an additional top dressing in June to a total of 140 kg ha^{-1} mineral nitrogen in the topsoil (0-0.30 m). In August, mineral nitrogen in the topsoil was supplemented to a total of 200 kg ha^{-1} nitrogen with an additional top dressing. The low nitrogen treatment received no additional nitrogen and no supplements.
The calcium treatment (indicated as high calcium) was applied as a root immersion treatment in a 3% aqueous solution of calciumchloride. Chicory heads were produced at three forcing seasons, early in November, intermediate in December and late in March. Chicory heads of cultivars Bea, Clause R2, Salsa and Tabor were produced under the low nitrogen and calcium treatment and produced in the intermediate and late forcing season. Chicory heads of cultivar Rumba were produced under both nitrogen and calcium treatments during all forcing seasons.
Cell wall extracts from freshly harvested chicory heads of the intermediate and late forcing season of cultivar Rumba were prepared according to McGuire and Kelman (1986) and Voragen (1980) by acid alcohol extraction of the cell walls from chicory pulp. Calcium content of freshly harvested chicory heads and of cell wall extracts of the heads from cultivar Rumba from the early and intermediate forcing seasons was measured by the Chemical Laboratory of the Research Institute for Agrobiology and Soil Fertility (AB-DLO) using a flame spectrophotometer.

Bacteria

Erwinia carotovora subsp. *carotovora* (Jones) Bergey *et al.* 1923 (*Ecc*) isolate nr. PD-1053 from witloof chicory cultivar "Flash" was used in these experiments. Cultures were grown at 27°C both in nutrient broth (NB) and nutrient agar (NA). Cells were directly harvested from broth or suspended in sterile water from agar plates using 2-3 day old cultures.

Suspensions were centrifuged for 10 minutes at 12000 rpm (Sigma centrifuge) and the pellet was suspended to obtain appropriate densities for inoculations. Densities were assessed by turbidity measurement using a colorimeter at 600 nm.

Inoculation of chicory heads and cell wall extracts

The outer leaves of harvested witloof chicory heads were removed aseptically and the heads were weighed. Chicory heads were surface sterilised with 70% ethanol and one leaf was removed using sterile equipment, causing a wound for artificial inoculation. The bacterial suspension (15 µl, $1.5*10^5$ cfu/ml) was applied to the wound. Inoculated chicory heads were placed in sterile plastic boxes, boxes were sealed and incubated at 25°C. Cell wall extracts of cultivar Rumba were suspended in Protein extract medium (PEM) prepared according to Klement (1990) with the omission of all carbon sources (2 gram cell wall extract in 25 ml PEM). Media were prepared separately for both calcium and nitrogen treatments of the cell wall extracts and inoculated with *Ecc* to a final density of $5*10^6$ cfu/ml.

Assessment of disease

The amount of naturally occurring softrot after forcing was assessed directly after forcing of the chicory heads of cultivar Rumba for the three forcing seasons during each of the three forcing seasons in 1994 until 1997 to a total of 12 assessed forcing seasons. The numbers of diseased and healthy heads were counted per treatment and the fraction of diseased heads (disease incidence) was used for statistical analysis. The amount of maceration in artificially inoculated chicory heads was measured daily until 4 days after inoculation. Rotting tissue was removed from the chicory heads with a sterile spatula. The macerated tissue and the remaining healthy tissue were weighed and disease severity was defined as the percentage of macerated tissue per chicory head. Two grams of macerated tissue were suspended in sterile water and the turbidity of the suspension measured in a turbidity meter at 600 nm. Samples of 15 µl were serially diluted and plated on tryptic soy agar (TSA) to count bacteria numbers present in the macerated tissue. Plates were incubated at 27° until colonies were clearly visible. Bacterial density was assessed daily and expressed as the number of cfu's per gram of macerated tissue.

Measurement of enzyme activity

Activity of pectolytic enzymes of *Ecc* was measured both in plant tissue and cell wall extracts from chicory heads from cultivar Rumba. The effects of the nitrogen and calcium treatments of the chicory roots on the activity of the enzymes were compared using plant tissue and cell wall extracts from freshly harvested chicory heads.

Measurement of enzyme activity in chicory heads inoculated with *Ecc*
After removal of the brown and rotting chicory tissue, a sample of the head of about 1 gr from the area between diseased and healthy tissue was cut from the chicory head. Samples were weighed, cut into small pieces and put in 2 ml sterile water (MilliQ). Samples were gently agitated to release intercellular fluids. For direct assessments the extracts were filter-sterilised with a 0.2 mm filter (Redrim, Schleicher and Schuell). Samples of the suspensions were used for acetone precipitation as described below.

Measurement of enzyme activity in artificial medium with cell wall extracts inoculated with *Ecc*

Samples of 3 ml from the liquid cultures were centrifuged at 12,000 rpm to remove bacterial cells and used directly for acetone precipitation. For acetone-precipitation, the samples were precipitated with 4 volumes of acetone at -20°C for one hour, centrifuged at 12,000 rpm for 10 minutes (Sigma centrifuge) and the pellet was washed twice in 80% acetone. Measurements were performed at pH 5 for polygalacturonase (PG) and at pH 8 for pectate lyase (PL). The pellet was suspended in the appropriate buffer (0.1M sodium acetate, pH 5 for PG or 50 mM Tris-HCl, pH 8 for PL). Presence of enzymes was verified at 235 nm in a spectrophotometer according to Lange and Knösel (1970). Polygalacturonase activity was assessed using the modified Nelson's assay (Easton and Rosall, 1985). Pectate lyase activity was measured at 235 nm in a spectrophotometer (Collmer *et al.*, 1988). Enzyme activity was measured 12, 24, 48 and 72 hours after inoculation in two replications per sampling time. One unit of activity of the enzymes was defined as the amount of enzyme liberating 1 µmol of reducing groups per min at 25 °C.

Statistical analysis

Disease incidence after forcing of witloof chicory Rumba was analysed after asin$\sqrt{}$-transformation of the data. Data from plots with visible symptoms of disease in the chicory heads (incidence > 0) were used for analysis. Analysis of variance was used to assess the effects of the forcing season and the nitrogen and calcium treatment on disease incidence. Data were pooled over the four years of the experiment. Tissue maceration of chicory heads, growth and enzyme activity of *Ecc* were measured daily until 4 days after inoculation for the artificially inoculated chicory heads of cultivar Rumba. All assessments were performed in two replications per nitrogen and calcium treatment per sampling time (n = 8). Bacterial densities were log – transformed. Differences between treatments were analysed using Tukey's Honestly Significant Difference (HSD) test at α = 0.05 unless stated otherwise. Disease severity in the tested cultivars was analysed per sampling day calculating the least significant difference (LSD) between cultivars. Enzyme activity of *Ecc* in artificial media with cell wall extracts from cultivar Rumba as a pectate source were measured daily until 4 days (72 hrs) after inoculation. The effect of the nitrogen and calcium treatments of the cell wall extracts were analysed using Tukey's Honestly Significant Difference (HSD) test at α = 0.05 (n = 8) unless stated otherwise.

Results

Disease incidence after forcing of the heads

The occurrence of bacterial softrot was monitored in chicory heads of cultivar Rumba directly after forcing of the roots of both nitrogen and calcium treatments. Bacterial softrot occurred during all three forcing seasons in the years 1994-1997, no significant differences were found for the disease incidence between years (Table 1).

The average disease incidence ranged from 0.35 in the early forcing season to 0.31 in the intermediate and late forcing season. Application of the calcium treatment reduced the average disease incidence with 8% in the early, with 31% in the intermediate and 23% in the late forcing season.

The nitrogen treatment of the roots increased the disease incidence with 6% in the early forcing and with 13% in the intermediate and late forcing season. The forcing season and the calcium- and nitrogen treatments had a significant effect on the disease incidence. Highest disease incidence was found for the low calcium - high nitrogen combination for all three forcing seasons. The treatment combination low calcium - low nitrogen resulted in a significantly higher disease incidence during the intermediate and the late forcing season, but not during the early forcing season.

Analysis of variance of the transformed disease incidence showed significant effects for the calcium and nitrogen treatments and for the forcing season (Table 2). Also the treatment combination calcium - nitrogen and the interaction between calcium treatment and forcing season were significant. This analysis indicates that disease incidence in chicory heads changed not only with the nitrogen fertilisation of the roots but also during the forcing seasons from early to intermediate and late forcing. Overall average disease incidence was highest during intermediate forcing (0.42) and lower during the other two periods (0.36).

Disease severity in several chicory cultivars

Chicory heads from several cultivars and forced from roots of the low nitrogen and calcium treatment showed a different disease severity for the cultivars (Figure 1). Disease severity increased exponentially for all cultivars during the incubation period. The disease progressed most rapidly in Salsa (significantly highest severity after 48 hrs) and slowest in Clause (significantly lowest severity after 96 hrs). After the final observation at 96 hrs, cultivars Tabor and Bea were macerated almost completely, cultivars Rumba and Salsa were macerated for more than 60% and cultivar Clause R2 only for 6%.

Table 1: Average disease incidence and standard errors after forcing of chicory heads from cultivar Rumba for three forcing seasons of witloof chicory with two calcium and two nitrogen treatments of the roots prior to forcing (incidence > 0, asin$\sqrt{}$ - transformed).

Calcium treatment	Nitrogen treatment	Forcing season		
		early	intermediate	late
High	High	0.33 ± 0.15 a[1]	0.33 ± 0.13 a	0.32 ± 0.10 a
	Low	0.36 ± 0.13 a	0.29 ± 0.10 a	0.30 ± 0.11 a
Low	High	0.40 ± 0.17 a	0.52 ± 0.20 bc	0.44 ± 0.16 b
	Low	0.34 ± 0.15 a	0.45 ± 0.19 b	0.36 ± 0.15 a

[1] Values in a column not followed by a common letter are significantly different at $p \leq 0.05$ according to Tukey's HSD test.

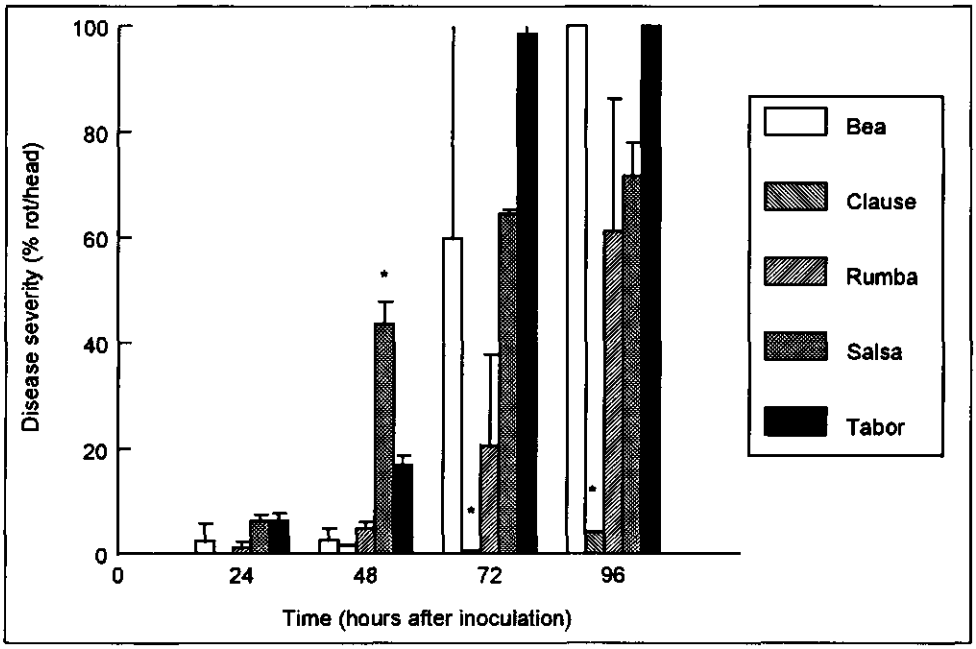

Fig. 1: Disease severity in witloof chicory heads after inoculation with *Ecc* (15 µl, $1.5*10^5$ cfu/ml) of several cultivars. Chicory heads were incubated at 27°C for 4 days and severity assessed daily.

Disease severity and enzyme activity of *Ecc* inoculated on chicory heads of cultivar Rumba

The progress of the disease in the chicory heads was different for the cultivars and the final observation of disease severity, 96 hours after inoculation, was significantly different for the chicory Clause R2 compared to the other cultivars. After the incubation period, disease severity was more than 50% in all cultivars except for Clause R2.

Table 2: Significance levels from ANOVA for the softrot incidence after forcing of chicory heads from cultivar Rumba for three forcing seasons during 1994-1997 for two calcium and nitrogen treatments of the roots prior to forcing (incidence > 0, asin√ - transformed).

Source	df	Disease incidence
Calcium	1	<0.000
Nitrogen	1	<0.000
Forcing season	2	0.005
Calcium * Nitrogen	1	0.007
Calcium * Forcing season	2	<0.000
Nitrogen * Forcing season	2	0.320
Error	759	

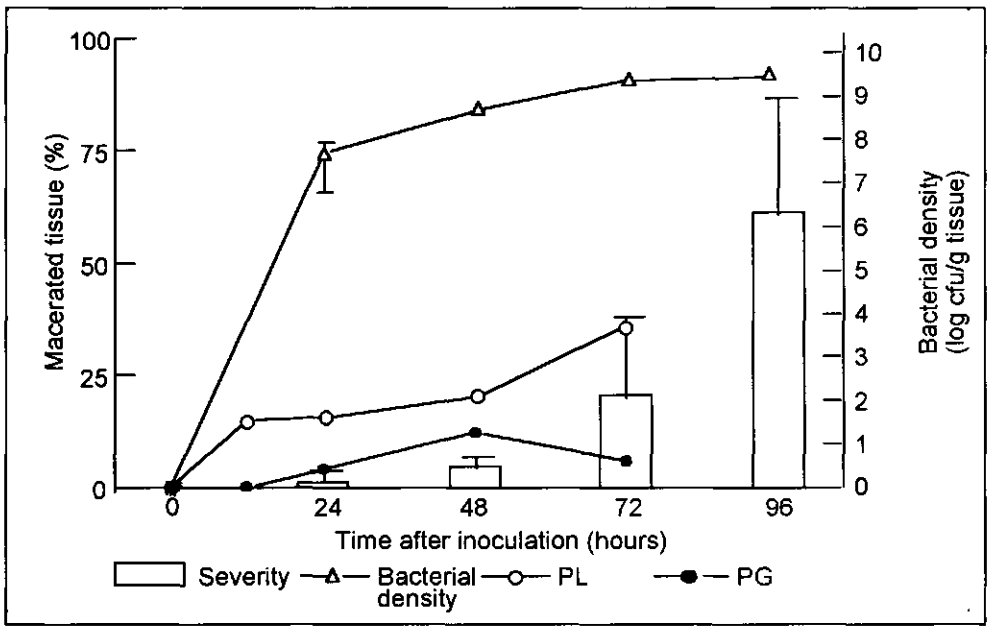

Fig. 2: Growth of *Ecc* (right y-axis) and severity (percentage of macerated tissue, left y-axis) of witloof chicory heads of cultivar Rumba from the low nitrogen and calcium treatment after artificial inoculation with *Ecc* (15 µl, $1.5*10^5$ cfu/ml). Activity of the pectolytic enzymes PL and PG (*100) is drawn on the left y-axis.

Chicory heads of cultivar Rumba from the low nitrogen and low calcium treatment were harvested. Healthy chicory heads were artificially inoculated with *Ecc* and the disease progress was monitored. Disease severity increased exponentially and coincided with an exponential growth pattern of the bacteria in the macerated tissue of the chicory heads (Figure 2). Tissue maceration of the chicory heads increased during the incubation period up to an average severity of 60% after 96 hours when the bacterial population reached its stationary phase at a density of $5*10^9$ cfu per gram macerated tissue. The activity of the two pectolytic enzymes PL and PG was monitored. PL activity was detected early)12 hpi). Levels did not increase substantially until 48 hpi. After this time a second strong increase in PL activity was observed. PG activity was first detected only at 24 hours after inoculation (hpi) and increased to a maximum level 48 hpi.
Between 48 and 72 hpi PG activity decreased by 50% in spite of a fivefold increase in bacterial density in the macerated tissue. Enzyme activity of *Ecc* grown in artificial media with cell wall extracts of cultivar Rumba. Cell wall extracts of chicory plants treated with both nitrogen and calcium treatments were added as a pectate source to artificial media inoculated with *Ecc*. We tested the effect of cell wall extracts of different origin and constitution on the activity of the pectolytic enzymes. The calcium content of the cell wall extracts was measured for the four treatment combinations. The calcium content of the cell wall extracts was significantly different for the two calcium treatments but was not influenced by the nitrogen treatment.

The calcium content of the cell wall extracts was on average 8.5 gr kg^{-1} and varied from 5.9 gr calcium kg^{-1} in cell wall extract from the high nitrogen and low calcium treatment combination to 10.9 gr kg^{-1} in cell wall extract from the low nitrogen and high calcium treatment combination. *Ecc* grown in artificial media with cell wall extracts showed an exponential growth pattern of the bacteria during the incubation period (Figure 3). Bacterial densities increased from $5*10^6$ cfu ml^{-1} 0 hpi to 10^{10} cfu ml^{-1} in the stationary state 48 hpi. The growth of *Ecc* grown in media with cell wall extracts was not significantly affected by the two nitrogen and calcium treatments of plants from which the cell wall extracts were made. The highest activity of PL coincided with the exponential growth stage of the bacteria 24 hpi and enzyme activity dropped when the bacteria reached the stationary growth stage, 72 hpi.
The transient course of the PL activity in cell wall extracts was different from the continuous increase of the PL activity in artificially inoculated chicory heads (Figure 2) but it was comparable to the PL activity patterns found in artificial media with various synthetic pectate sources (data not shown).
The activity of PL was higher using cell wall extract from the high nitrogen treatment than from the low nitrogen treatment (Table 3) but decreased in both nitrogen treatments during the incubation period. PG activity reached the highest activity 48 hours after inoculation. The activity of both PL and PG of *Ecc* grown in cell wall extracts from the low nitrogen treatment and 24 hours after inoculation differed significantly for the two calcium treatments.

Discussion

Ecc is a softrot pathogen and its virulence is related to the amount of macerated tissue caused by the pathogen caused by the production of extracellular pectolytic enzymes (Pérombelon & Salmond, 1995). During the infection process, several steps occur before softrot symptoms appear. The bacteria present in the intercellular spaces of the chicory tissue produce pectic enzymes on a constitutive and rather low level. These exo-enzymes cleave structural pectic polymers in the plant cell wall and middle lamella, facilitating the penetration and colonisation of the tissue (Collmer & Keen, 1986). Products of the pectate digestion will activate a regulatory mechanism in the bacteria which stimulates synthesis of extracellular pectolytic enzymes (Alghisi & Favaron, 1995).

Table 3: Bacterial enzyme activity in cell wall extracts from chicory heads cultivar Rumba for two nitrogen and two calcium treatments of the roots prior to forcing

Time (hours) after inoculation	Nitrogen - treatment	PL activity (µmol/min)		PG activity (µmol/min)	
		Calcium treatment		Calcium treatment	
		High	Low	High	Low
24	High	0.35	0.34	0.58	0.45
	Low	0.31*[1]	0.16*	0.57*	0.31*
48	High	0.14	0.22	0.84	1.02
	Low	0.06	0.09	0.94	0.70
72	High	0.004	0.004	0.64	0.63
	Low	0.005	0.004	0.64	0.57

[1] Enzyme activities are different for the calcium treatments at $p < 0.05$ according to Tukey's HSD test.

We hypothesised that cultural practices such as the nitrogen and calcium treatments studied here, should not only influence disease incidence and severity in the crop but also influence the enzymes regulating the pathogenicity directly. Free calcium in the form of bivalent ions is necessary for the activation of PL but inhibits PG (Pagel & Heitefuss, 1990). Calcium also influences the pectate polymers in the plant cell walls and middle lamella (Barras *et al.*, 1994). Calcium ions are incorporated in the pectate molecules and form an "egg-box" structure which is less accessible for pectolytic enzymes than calcium-free pectate (McGuire & Kelman, 1986). The enzyme activity of the constitutively produced enzymes PG and PL could therefore be an indicator for disease severity. The transient course of the PL activity in liquid culture containing cell wall extracts was different from the continuous increase of the PL activity in artificially inoculated chicory heads (Figure 2 and 3).
The continuous increase of PL activity is caused by the transcription of several gene families of PL enzymes. These gene families are regulated differently in vitro and in planta. Yang *et al.* (1992) have shown that accumulation of mRNAs for PL in *Ecc* was sequential and correlated with rotting of the plant material and did not occur in liquid culture of *Ecc* using polygalacturonic acid as a pectate source. In cell wall extracts, the temporal pattern of PL activity but not of the PG activity differed from the enzyme activity as measured in chicory heads. The lack of metabolites, especially 2-keto-3-deoxygluconate (KDG) directly interfering with the repression of genes encoding for pectate lyase so-enzymes (Pérombelon & Salmond, 1994).
The growth media containing only cell wall extracts provide only one carbon-source for the softrot bacteria, whereas in macerating tissue the cell walls rupture providing nutrients for the bacteria in addition to the cell wall fragments. The induction of pectolytic enzymes is known to vary between studies in vitro and in planta (Bateman & Millar, 1966). Several plant inducible iso-enzymes of PL have been identified in *Erwinia chrysanthemi* which are regulated independently from the pectate inducible iso-enzymes (Kelemu & Collmer, 1993). Studies of Zucker *et al.* (1972) and McMillan *et al.* (1993) have shown that potato extracts enhance PL activity in *E. carotovora*.
Using cell wall extracts as a growth substrate for *Ecc*, the additional stimulation of the enzyme activity by plant extracts is lacking. Barras (1994) showed that softrot *Erwinia's* produce other iso-enzymes of PL in planta than in vitro and extracellular levels of PL are barely detectable in basal salt media but highly increased when adding plant extracts to the medium (Murata *et al.*, 1988). PG is inhibited by plant extracts of canola, wherein the inhibition is directly related to the calcium contents of the plant extracts (Annis & Goodwin, 1997).
The forcing season, nitrogen and calcium treatment had a significant effect on disease incidence after forcing. During the intermediate and late forcing seasons, more disease was found in chicory heads from the high nitrogen and low calcium treatments. Using artificially inoculated chicory heads to measure the activity of the pectolytic enzymes PG and PL, we found no differences in the enzyme activity between the nitrogen and calcium treatments.
Nitrogen levels in the crop are known to influence softrot severity (Bartz *et al.*, 1979; Canaday & Wyatt, 1992; Carballo *et al.*, 1994). Nitrogen fertilisation increases the dry matter content of broccoli (Everaarts, 1994) and has a direct effect on the cell wall composition, especially the esterification of pectate in the cell walls. In addition, nitrogen fertilisation interacts with the production of plant defence substances as phenols and it inhibits calcium uptake (Reerink, 1993).

Different chicory cultivars showed different sensitivity to tissue maceration. At the end of the incubation period, severity varied from 100% macerated tissue in Tabor to 6% in Clause R2. The high variation between replications during this experiments is a point of concern. Even in large experiments performed under highly conditioned circumstances the variance of the results is such that only weakly significant results appear. Part of the variation can be explained by the time delayed effects of the applied treatments.

Nitrogen fertilisation is applied during the root production, two to 8 months before forcing. Calcium immersion of the roots is applied directly after harvest of the roots in September. The effects both of nitrogen and calcium on the softrot incidence and the disease severity after forcing of the chicory heads are most probably connected with other factors, especially the physiological condition of the chicory roots during forcing. More research is needed to link this differences to differences in cell wall composition. Pérombelon and Salmond (1994) found a correlation between the resistance of potatoes against bacterial softrot to the composition of the plant cell walls. The pathogenicity of *Ecc* could be reduced when either the bacterial multiplication or the activity of the pectolytic enzymes was reduced. As the first aspect is directly linked to the second aspect of enzymatic breakdown of the cell walls, cultural measures known to reduce bacterial softrot without affecting the bacterial infestation and infection, are most likely to interact on the level of enzymatic breakdown of the cell walls.

The resistance of witloof chicory cultivars against bacterial softrot may be linked to the ability of chicory cultivars to incorporate calcium in the cell walls analogous to potato cultivars. Potato cultivars resistant to bacterial softrot had consistently higher amounts of calcium in cell wall preparations than more susceptible cultivars and increased levels of galacturonic acid in cell walls (McGuire & Kelman, 1986). An increasing calcium fertilisation of the tubers increased the deposits of calcium in the cell walls and improved the stability of the cell. They concluded that as calcium inhibits the primary pathogenicity factor of softrot bacteria, the pectolytic enzymes, calcium uptake may be at least partially responsible for resistance against bacterial softrot.

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Chapter 4

Water and temperature relations of softrot bacteria: growth and disease development.

Water and temperature relations of softrot bacteria: growth and disease development.

B M Schober and J C Zadoks

Summary

The effect of the water availability and the temperature of the growth substrate on growth and disease development of softrot bacteria was studied using artificial media and plant material. Water availability was measured as the osmotic potential of a solution (ψ_{osm}) and was assessed for solutions of PEG4000 and KNO_3 as artificial osmotica and for plant tissue of chicory heads. Growth of softrot bacteria was found at water potentials from ψ = -0.12 MPa to ψ = -8.0 MPa but the lag phase of the growth curve increased with decreasing water potential. The relative growth rates of the three softrot pathogens showed a sigmoidal relationship with water potential, the relative growth rates decreasing rapidly at water potentials lower than ψ = -1.5 MPa. The water potential of harvested chicory heads decreased with storage time of the harvested crop but was still within the growth limits for softrot bacteria. In relation to temperature, the relative growth rate of *Ecc* was highest at 10°C, of *Eca* at 15°C and of *Pm* at 5°C. Chicory heads of two chicory cultivars, Rumba and Salsa, inoculated with *Ecc* had a significantly higher disease severity at 30°C (0.72 for Rumba and 0.47 for Salsa) than at lower or higher temperatures. In conclusion, temperature and water availability during forcing of chicory are not factors limiting populations of softrot bacteria. Possibilities for crop protection thus only avail during chicory root storage. During storage a high death rate comined with a low growth rate of the softrot bacteria may result in a decrease of bacterial populations below the minimum densities needed for infection during forcing of the chicory heads.

Introduction

Bacterial softrot of witloof chicory is a major spoilage disease during forcing and harvest of the chicory crop. The growing chicory heads can develop a slimy rot during the last days of forcing which may induce losses up to 60% of the total crop. This softrot is caused by several phytopathogenic bacteria, of which *Erwinia carotovora* subsp. *carotovora* (Jones) Bergey *et al.* 1923 (*Ecc*) and *Pseudomonas marginalis* (Brown) Stevens 1925 (*Pm*) are the most common in the Netherlands (Schober & Zadoks, 1998).

Erwinia carotovora subsp. *atroseptica (Eca)* causes bacterial softrot in chicory in Belgium and France (Vantomme *et al.*, 1989). Epidemics of softrot are determined by three factors, the population of plants, the population of the pathogens and the environment (Goto, 1992). Temperature and humidity are important factors in disease development and severity. Water availability is considered to be one of the most important determinants for bacterial growth and development (Colhoun, 1973). Both bacterial multiplication and pathogenicity are temperature and water dependent. *In vitro* studies indicated the optimum values for softrot bacteria of temperature (Goto, 1992; Dealto & Surico, 1982) and water availability (Mildenhall *et al.*, 1981; Membré & Burlot, 1994).

In potato, the minimum inoculum density needed for infection is temperature dependent and free water is required for disease development. Pathogenicity of softrot *Erwinia's* is directly related to water availability in the crop tissue. The susceptibility of plant tissue increases with water potential (Pérombelon & Salmond, 1995) and the crop physiology can change at low temperatures resulting in plant tissue which is more susceptible to softrot (Sellam *et al.*, 1980). Many pseudomonads are psychrotrophic, and bacterial rot of vegetables caused by *Pm* most commonly occurs from autumn to early spring when plants suffer from frost damage (Goto, 1992), or during cold storage and transport of the harvested product (Liao, 1989).
Witloof chicory roots are grown in a temperate climate. Chicory is sown in spring and the roots are harvested in late autumn. The harvested roots are stored in cells at 0-0.5°C and at a high relative humidity for at least four weeks and up to six months. For chicory head production, the roots are sorted into trays and placed in forcing cells with an air temperature of 15-20°C and a water temperature of the hydroponics system of 16-18°C (Le Guern *et al.*, 1992).
The roots are forced to produce an etiolated chicory head in 3 to 4 weeks, the physiology of the crop may be altered to a state more conducive to infection and spread of bacterial softrot (Pérombelon & Salmond, 1995). Environmental conditions during root production and storage are favourable for the growth of softrot bacteria and the crop may be more susceptible to softrot due to the environmental conditions throughout the entire production process.
This paper examines the growth characteristics of softrot bacteria isolated from witloof chicory heads and studies the effect of temperature and water availability on the multiplication of softrot pathogens and disease development using artificial media and plant material.

Material and Methods

Bacterial inoculum

Type strains PD819 *Erwinia carotovora* subsp. *atroseptica* (van Hall 1902) Dye 1969 (*Eca*), PD1053 *Erwinia carotovora* subsp. *carotovora* (Jones) Bergey *et al.* 1923 (*Ecc*), both isolated from the chicory cultivar Flash, and PD980 of *Pseudomonas marginalis* (Brown) Stevens 1925 (*Pm*), isolated from a Belgian chicory sample, have been used in all experiments. Pure cultures were maintained on Nutrient Agar (NA, Oxoid) at 27°C. For inoculation, a single colony from a 48h old culture was suspended in sterile Nutrient Broth (NB, Oxoid). To determine inoculum density, the absorption (A) was measured at 600 nm in a colorimeter (Colorimeter 257, CIBA-Corning) and the density was adjusted to the appropriate densities by adding sterile NB according to the formulas
#cfu $ml^{-1} = 9.4*10^9 * OD_{600}$ for *Eca* and *Ecc* and #cfu $ml^{-1} = 4.6*10^9 * OD_{600}$ for *Pm*.

Water availability experiments

Measurement of water potential

Water availability can be expressed as water activity a_w (Scott, 1957), related to relative humidity by a_w = %RH/100, or as water potential ψ (Duniway, 1976), related to a_w by $\psi = (RT/V)\ln(a_w)$, where R is the gas constant (8.314), T is the absolute temperature (K) and V is the mole volume ($m^3\ mol^{-1}$) of water. The water potential ψ is the sum of three partial potentials, the pressure component ψ_p caused by the pressure of water molecules onto the bacterial cell, the matrix potential ψ_m caused by the chemical or physical binding of water to large molecules and particles and the osmotic potential ψ_{osm} caused by the binding of water molecules to electrically charged particles like ions and sugars.

The total water potential is expressed as $\psi_{tot} = \psi_{osm} + \psi_m + \psi_p$ (Papendick & Campbell, 1981). In solutions, the matrix and the pressure components are zero, so the total water potential equals the osmotic potential of the solution $\psi_{tot} = \psi_{osm}$. The unit of measurement is Pascal (MPa). The osmotic water potential was measured by an Osmometer (Osmomat 030, Gonotec) using the freezing-point depression caused by salts and sugars in a solution. The water potential of 50 µl samples from the solutions was calculated with a calibration curve as ψ_{osm} = osmolarity * T * R * weight (sample) * 1000 where R is the gas constant and T is the absolute temperature. Osmolality is measured in osmol kg^{-1} and weight in grams.

Experiments in liquid culture

KNO_3 and PEG4000 were used as osmotica (Table 1). Stock solutions of 500g l^{-1} PEG4000 and 10.1g l^{-1} KNO_3 in NB were prepared, autoclaved and used for the solutions. The water potentials of the solutions were determined using a calibration curve. The water potential of NB itself after autoclaving was -0.12 MPa.

Erlenmeyer flasks containing 50 ml were inoculated with *Eca*, *Ecc* or *Pm* to a final density of $5*10^4$ cfu ml^{-1}. Five flasks per water potential and per pathogen were incubated in an orbital incubator (Gallenkamp), 120 revolutions per minute, at 27°C. Growth of the bacterial cultures was periodically measured in two replicates per flask as the optical density of the cultures at 600 nm (OD_{600}) or by plating samples of the cultures (four per flask) on Nutrient Agar (NA, Oxoid) using a Spiralplater (Spiraltech) and counting the number of colonies in 50 µl samples.

Measuring water potential in plant material

After harvest, chicory roots were divided into two lots. One lot received a Calcium immersion treatment using a 30g l^{-1} solution of Calcium chloride (Calcium), the other not. Both samples were treated identically from this point onward. Chicory heads were forced at the Experimental Station for Arable Farming and Vegetables (PAV) under circumstances described elsewhere (Schober & Zadoks, 1998a) and the harvested heads were packed and placed in cold storage at 5°C directly after harvest.

In both lots, with and without Calcium, water potential of chicory heads was measured directly after harvest, and after one and two weeks of storage. To measure water potential of both leaves and leaf stalks, the fleshy leaf stalks of the chicory heads were separated from the leaves.

From all plant parts, samples of 1g were macerated with a glass rod in 5 ml MilliQ and shaken vigorously. Samples were left for 15 minutes to release cell contents into the liquid. The water potential of 50µl aliquots of the suspensions was measured twice using the Osmometer.

Temperature experiments

Experiments in liquid cultures
Erlenmeyer flasks containing 50 ml NB were inoculated with *Eca*, *Ecc* or *Pm* to a final density of $1.5*10^3$ cfu ml^{-1}. Two flasks per temperature for each pathogen were incubated in an orbital incubator (Gallenkamp) at 120 revolutions per minute at five temperatures, at 0°C, 5°C, 10°C, 15°C and 20°C. From each of the two replications per temperature, four 50µl samples were taken to determine bacterial densities, as described above. Bacterial densities were determined periodically until 108 hours after inoculation.

Experiments *in planta*
Chicory heads of the cultivars Rumba and Salsa were surface sterilised with 70% ethanol and cut lengthways with a sterile knife. The two halves were weighed and placed together in a plastic box with the cut surfaces of the chicory halves facing up. The box was prepared by lining the bottom with sterile filter paper moistened with MilliQ and 0.02% NaN_3 to keep humidity near to saturation and to prevent spread of the bacteria. The halves were inoculated with 50 µl of *Ecc* at a density of $5*10^7$ cfu ml^{-1} using droplet inoculation. Aliquots of the bacterial suspension were distributed evenly in small droplets and spread further over the cut surface of the chicory halves using a sterile loop. Boxes were sealed with a lid and placed in an incubator. For each temperature (10°C, 15°C, 20°C, 25°C, 30°C and 35°C) two boxes were incubated. After three days the macerated tissue was removed from each chicory half and weighed. The remainder of each half was weighed too. Disease severity was expressed as the weight of the macerated tissue relative to the total weight of macerated and unmacerated tissue per half. Severity was averaged per box, the basic statistical unit.

Statistical analysis
Average bacterial densities, average population growth rates, average disease severity and their standard deviations were calculated for each sampling time. Data were log -transformed for bacterial density and √-transformed for disease severity when necessary and differences between treatments were analysed using Tukey's honestly significant difference test (HSD).

Table 1. Water potential measured as the osmotic potential of solutes in Nutrient Broth

PEG4000 (mol kg^{-1})	ψ (MPa)	KNO_3 (mol kg^{-1})	ψ (MPa)
0.000	-0.12	0.00	-0.12
0.025	-0.50	0.30	-0.45
0.050	-1.50	0.40	-0.50
0.075	-3.50	0.60	-0.55
0.100	-5.50	0.70	-0.60
0.125	-8.00	0.80	-0.65

The relative growth rates (Zadoks & Schein, 1979) were calculated for the exponential phase of population growth of all *in vitro* experiments and plotted against water availability or temperature. Sigmoidal non-linear models were fitted to describe the relationship between relative growth rate and water potential using the statistical package SPSS 6.1 (1994).

Results

Water availability

The water availability of the substrate, expressed as the water potential, had a significant effect on bacterial growth, relative growth rate and disease development. Comparison of the bacterial densities 32 hours after inoculation showed significantly lower densities at ψ < -3.5 MPa for all pathogens with two significantly different groups for *Eca*, four groups for *Ecc* and three groups for *Pm* (Table 2). The lag phase of *Ecc* increased from 4 hours at ψ = -0.12 MPa to 12 hours at ψ = -8.0 MPa. During the lag phase the decline of the bacterial density varied from nil at ψ = -0.12 MPa to more than a hundredfold at ψ = -8 MPa (three values shown in Figure 1). Stationary population densities, attained at about 36 hours after inoculation, varied from 10^{10} cfu ml^{-1} at ψ = -0.12 MPa to about 10^{8} cfu ml^{-1} at ψ = -8.00 MPa. Bacterial densities were highest at ψ = -0.12 MPa and lowest for ψ = -8.0 MPa.

To measure the effect of the water potential on the relative growth rates of the three pathogens, curve fitting was applied using non linear algorithms for the general equation y = a + b/(1 + exp(-(x-c)/d)) to the relative growth rate and water potential. Curve fitting resulted in a sigmoidal relationship with a coefficient of determination of r^2 = 0.996 for *Ecc*, r^2 = 0.98 for *Eca* and r^2 = 0.87 for *Pm* (Figure 2). The relative growth rate decreased most rapidly between ψ = -0.12 MPa and ψ = -3.5 MPa. The relative growth rate r of all three pathogens decreased by a fivefold from r = 0.05 h^{-1} at ψ < -3.5 MPa to r = 0.25 h^{-1} at $\psi \geq$ -0.5 MPa. Curve fitting for the relative growth rates of *Ecc* under different ψ using KNO_3 as the osmoticum showed a sigmoidal relationship with r^2 = 0.78 (n = 20, p<0.05) comparable to the relative growth rates when using PEG4000 as the osmoticum.

Table 2. Average density (log cfu ml^{-1}) of bacterial cultures of the softrot pathogens *Ecc*, *Eca* and *Pm* 32 hrs after inoculation at various water potentials n liquid culture of Nutrient Broth inoculated to an initial density of 5*10^4 cfu ml^{-1}

Water potential (MPa)	Average density after 32 hrs (log cfu ml^{-1})		
	Eca	*Ecc*	*Pm*
-0.12	8.03a	7.94a	7.33a
-0.50	8.98a	7.78a	7.37a
-1.50	8.63a	6.05b	6.84b
-3.50	4.57b	5.60c	5.08c
-5.50	4.53b	5.34cd	5.04c
-8.00	4.49b	4.88d	4.99c
	(19 df)	(19 df)	(19 df)

[a] Values in a column not followed by a common letter are sinificantly different at p ≤ 0.05 according to Tukey's HSD test.

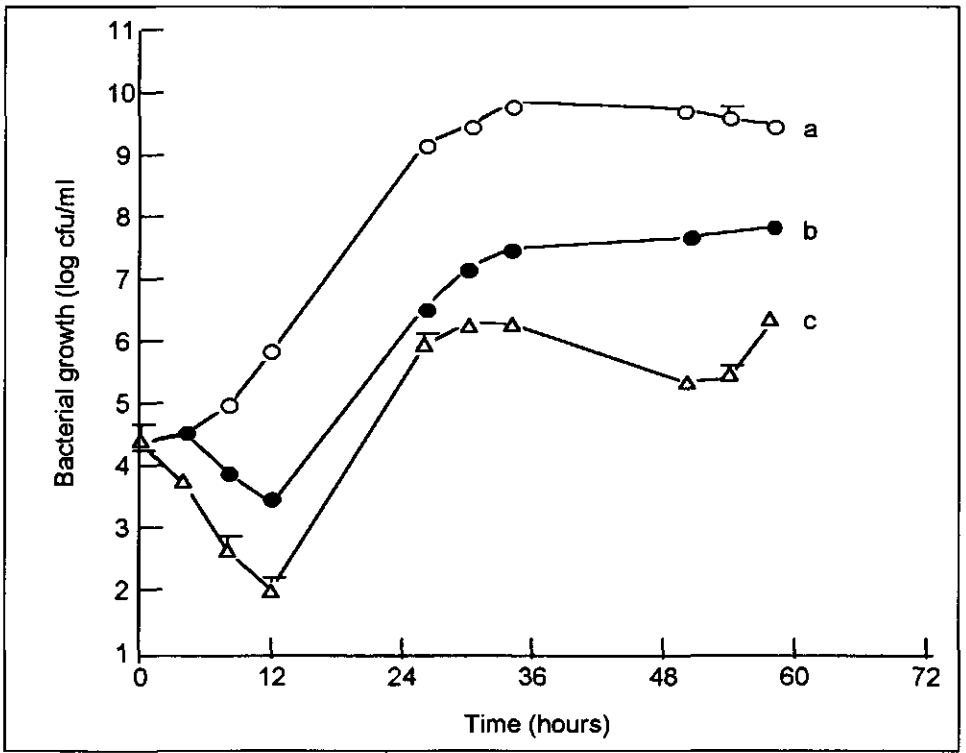

Fig. 1: Growth of *Ecc* as log cfu ml^{-1} at three different water potentials (a = -0.12 MPa, b = -3.50 MPa and c = -8.00 MPa) in liquid culture of Nutrient Broth with PEG4000 as the osmoticum at 27°C. Data points are averages of 5 replications per water potential.

Ecc showed almost no growth at $\psi \leq$ -3.5 MPa using PEG4000 and showed no growth at all at ψ = -0.65 MPa using KNO_3 as an osmoticum (Figure 3).

Water availability *in planta*

The water potentials measured in the plant parts (ψ = -0.12 to -0.17 MPa) were within the limits for bacterial growth as found *in vitro*. Storage of the harvested heads changed the water potential in the leaf parts (Table 3).The Calcium-treatment of the chicory roots did not affect the water potential in the heads significantly after one week of storage. After the second week of storage, plant parts with calcium treatment had significantly higher water potential than those without such a treatment, suggesting a better water retention with than without Calcium.

Temperature

Growth of bacteria *in vitro*

The type-strains representing three softrot causing bacterial species did behave similarly over the range of temperatures used for commercial chicory production.

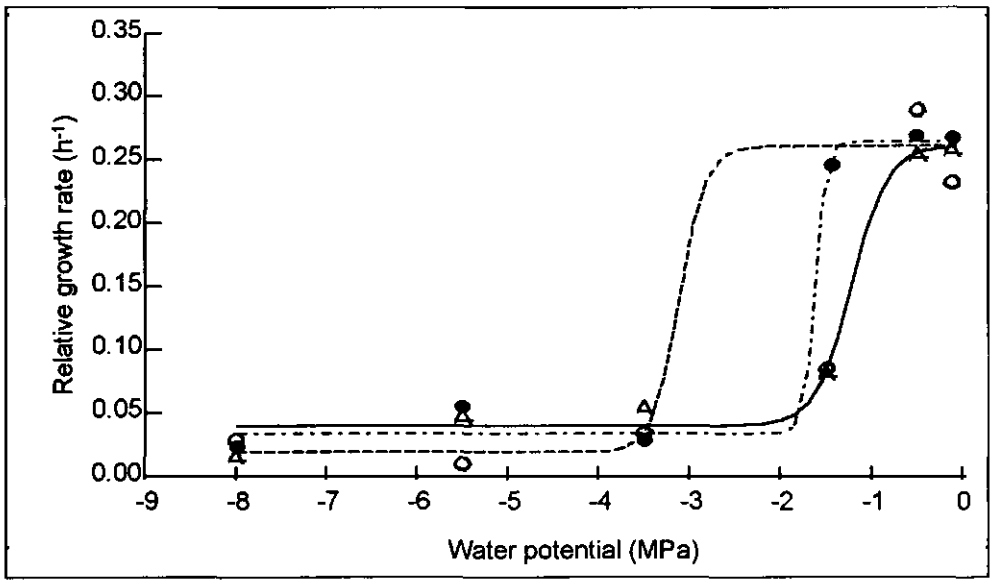

Fig. 2: Relative growth rates (per hour) of the exponential growth stage for the three softrot pathogens, *Ecc* (solid line), *Eca* (dashed line) and *Pm* (dotted line) at different water availability (MPa) in liquid cultures of Nutrient Broth with PEG4000 as the osmoticum at 27°C. Data points are averages of 5 replications per water potential. LSD (0.05) between water potential treatments is 0.05 for *Eca*, 0.15 for *Ecc* and 0.03 for *Pm*.

Table 3. Water potential in chicory heads after harvest and after one and two weeks of storage at 5°C.

Plant part	Calcium	Water potential of the tissue (MPa)		
		after harvest	after 1 week storage	after 2 weeks storage
Leaf	0	-0.17a[a]	-0.13a	-0.17a
	1	-0.15a	-0.12a	-0.15b
Stalk	0	-0.17a	-0.16a	-0.17a
	1	-0.17a	-0.14a	-0.14b

[a] Values in a column not followed by a common letter are significantly different at $p<0.05$ according to Tukey's HSD test (Data are pooled results with $n = 4$).

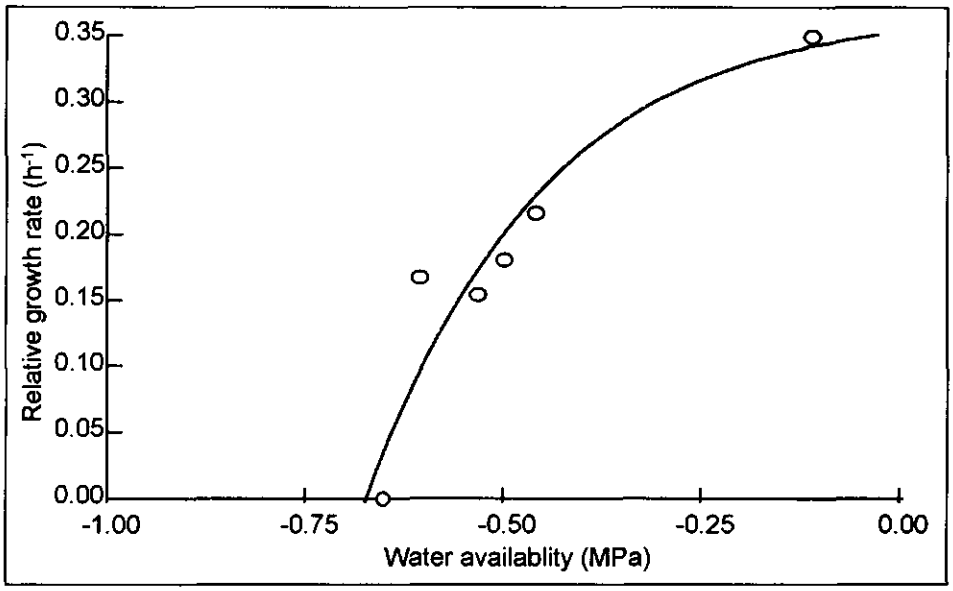

Fig. 3: Relative growth rates (per hour) of the exponential growth stage for the softrot pathogen *Ecc* at different water availability (MPa) in liquid cultures in Nutrient Broth with KNO3 as osmoticum at 27°C. Data points are averages of 5 replications per water potential. LSD (0.05) between water potential treatments is 0.12.

Though these strains tend to show high r-values at 5° - 15°C, we did not find significant differences between the temperatures. The relative growth rate of *Ecc* was highest at 10°C, of *Eca* at 15°C and of *Pm* at 5°C and was low at 0°C and 20°C for all three pathogens (Figure 4). *Ecc* and *Pm* responded similarly to temperature in liquid culture. For *Ecc* and *Pm*, bacterial densities at 0°C were lowest and significantly higher at 20°C than at temperatures ≤ 10°C. The density of *Eca* did not show significant differences over the temperature range from 0 to 20°C (Table 4).

Table 4. Average density (log cfu ml^{-1}) of bacterial cultures of the softrot pathogens *Ecc*, *Eca* and *Pm* 36 hrs after inoculation at various temperatures in liquid culture of Nutrient Broth inoculated to an initial density of $5*10^4$ cfu ml^{-1}.

Temperature (°C)	Average density after 36 hrs (log cfu ml^{-1})		
	Eca	*Ecc*	*Pm*
0	3.60a	3.55a	3.62a
5	3.66a	4.18ab	3.94ab
10	3.80a	4.37ab	3.96ab
15	4.00a	5.30bc	4.97bc
20	3.94a	5.88c	5.28c

[a] Values in a column not followed by a common letter are significantly different at $p \leq 0.05$ according to Tukey's HSD test (Data are pooled results with n = 8).

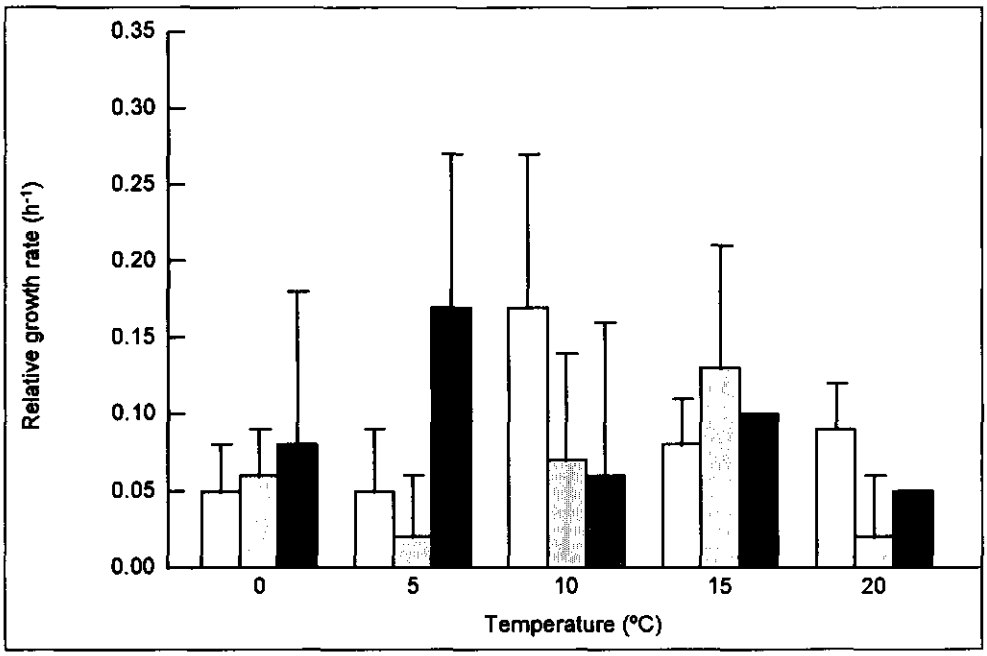

Fig. 4: Relative growth rates (per hour) of the exponential growth stage for the three softrot pathogens *Ecc* (white bars), *Eca* (light grey bars) and *Pm* (dark grey bars) at different temperatures (°C) in liquid cultures in Nutrient Broth. Data points are averages of 2 replications per temperature treatment.

Disease severity *in planta*

Disease severity in cultivar Rumba showed an optimum at 25°C to 30°C, was nil at 40°C and at ≤ 5°C, and negligible at 10°C and 15°C (Figure 5). Disease severity in cultivar Salsa followed roughly the same pattern but at a lower level. At 25°C and 35 °C, cultivar Salsa was significantly less diseased ($p = 0.048$, Tukey's HSD) than cultivar Rumba.

Discussion

Water potential

Bacteria react to decreasing water potentials by an attempt to regain equilibrium with the environment. The adaptation mechanisms vary between the bacterial species. *Erwinia* and *Pseudomonas* species belong to class II according to Harris (1981), with inducible solutes to counteract water stress. Growth of these bacteria is independent of the solute causing the water stress. Own results of the relative growth rates of *Ecc* using PEG4000 and KNO_3 as solutes are in accordance with Harris' data.

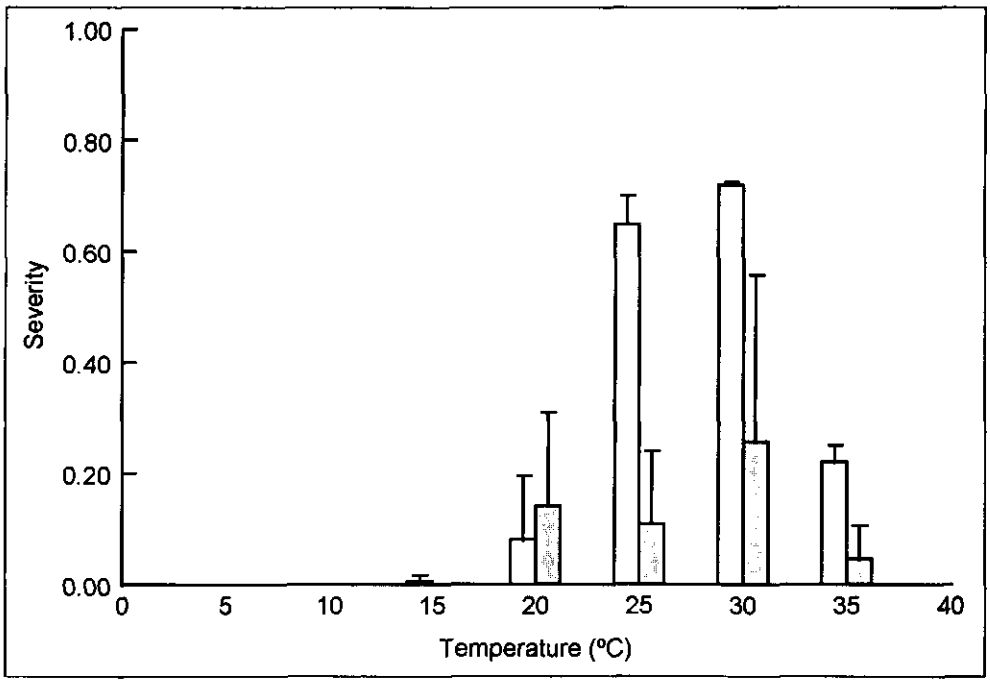

Fig. 5: Disease severity caused by *Ecc* after inoculation (10^6 cfu ml^{-1}) of witloof chicory heads cv. Rumba (white bars) and cv Salsa (grey bars) as the fraction of macerated chicory tissue per chicory head (severity) after 48 hrs incubation at different temperatures. Data points are averages of 2 replications per temperature treatment.

Survival of the bacteria (i.e. without a measurable increase of the bacterial population) and population growth have different optimum curves for water availability of the substrate. Bacterial growth without an adaptation period to the environment requires water activity and temperature near the optimum value for the particular species, survival is possible at conditions that are more adverse. Water potentials which cause water stress will increase the lag phase or cause an initial decline of the bacterial population (Scott, 1957) as found in our experiments.

Survival of the bacteria is possible in more extreme circumstances than required for growth and will evolve into growth as soon as the bacteria have adapted to the actual water stress. This adaptation is possible when osmotica are produced by the bacteria (Harris, 1981). After adaptation the bacteria developed relative growth rates which are comparable to the growth rates under optimal conditions at ψ = -0.12 MPa (Figure 1).

Optimal survival conditions are the result of several environmental conditions within the requirements for survival and not the combination of many optimal factors and one limiting factor. Interactions of the effects of temperature and water availability for the metabolism of micro-organisms are to be expected. These interactions may be synergistic or antagonistic. In experiments of Dealto and Surico (1982), *Pm* could survive at ψ = -0.80 MPa at temperatures under 10°C, but not at the same water potential and its optimum temperatures of circa 27°C. For the growth of the bacterial populations, the substrate has to be water-soaked or nearly so.

Water activities lower than a_w = 0.95 (i.e. ψ = -1.5 MPa), not suitable for growth but still allowing survival of the bacteria in a latent state, are common in the soil and on many plant surfaces. The bacterial populations will not increase under these circumstances but they can survive unless other environmental factors are unfavourable for survival (Hayward, 1974). Growth of and infection by pathogenic bacteria will occur only in plants or in wounded tissue where water is available, but survival is possible on plant surfaces and in the soil, also under conditions found during the production process of witloof chicory.

Temperature

The temperature influences both bacteria and plants. In general, *Eca* is more pathogenic at lower temperatures than *Ecc*, but optimum and lethal temperatures vary with geographical latitude for either pathogen. In north-western Europe, the optimum temperature for pathogenicity is circa 20°C for *Eca* and 25°C for *Ecc* (Pérombelon & Salmond, 1995).

In witloof chicory, *Eca* makes no major contribution to soft rot incidence in The Netherlands. *Ecc* is probably the only causal agent from the *Erwinia*-group found in The Netherlands. *Ecc* is adapted to low temperatures, isolates from potato (Pérombelon & Salmond, 1995) survive at 0-5°C in the stationary state (which implies low but positive growth rates) and they have a broader optimum temperature range varying from 20-30°C. Isolates from witloof chicory used in our experiments had lower optimum temperatures (between 15°C and 20G) than isolates from potato but had comparable growth characteristics at low temperatures. Temperatures during the three witloof chicory production stages, including the cold storage of harvested roots, are within the limits for growth and survival of the softrot pathogens.

Our experiments have confirmed that temperature and water availability during chicory production, from root production to storage and forcing, are well within the range needed for survival, growth and infection of softrot bacteria. During the root production in the field, temperatures and relative humidity allow multiplication of softrot bacteria (Schober & Zadoks, 1998b). Even under the most adverse conditions during root storage, the softrot bacteria can survive although population growth rates will be minimal (Schober & Zadoks, 1998c).

During the forcing of the chicory heads, temperatures and water availability are optimal for the softrot pathogens with ambient temperatures around 20°C and water available on the crop. Intervention for crop protection thus is only possible during root storage, where a prolonged storage period may result in a decrease of the population density of softrot bacteria well below the minimum density needed for infection.

Acknowledgements

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Chapter 5

Development of populations of softrot bacteria on witloof chicory leaves during root production in the field.

Development of populations of softrot bacteria on witloof chicory leaves during root production in the field.

BM Schober and JC Zadoks

Summary

During a 4-year field experiment at the Experimental Station for Arable Farming in Lelystad, the Netherlands, witloof chicory plants, grown for root production, were surveyed for the presence of epiphytic or saprophytic populations of the softrot bacteria (*Erwinia carotovora* and *Pseudomonas marginalis*) on their leaves. Isolates of pectolytic bacteria collected from the leaves were identified and the influence of environmental variables was assessed testing the hypothesis of an increase of the number of isolates on plants in the field. A significant linear correlation (R^2 = 0.47, n = 20, p = 0.002) was found for the regression of the number of pectolytic isolates on time (month) and temperature (monthly average). The average monthly growth rate (r) of the softrot bacteria was significantly greater than zero during the field season, indicating an increase of the populations of softrot bacteria. The ratio between the number of *Erwinia* and *Pseudomonas* isolates changed during the growing season from a predominance of pseudomonads from May until July to an equal ratio from August until harvest. The number of isolates of *Erwinia carotovora* was negatively correlated with total global radiation. The increase in the number of *Ecc* later in the growing season was attributed to protection from high radiation levels and adverse temperatures in the microclimate under the canopy.

Introduction

Bacterial softrot is a major disease of witloof chicory during forcing of the roots, the last production stage of the edible product. The production of chicory heads is divided into three stages, 1) the root production on the field, 2) the cold storage period of the harvested roots and 3) the forcing of the roots producing the chicory heads. These heads can develop a slimy rot caused by softrot bacteria. As studies have shown (Chapters 6 and 7), the bacteria are carried into the storage and forcing stages on the leaf stubs of the roots from stage 1.

The causal organisms include two common bacterial species, *Erwinia carotovora* subsp. *carotovora* (Jones) Bergey *et al.* 1923 (*Ecc*) and *Pseudomonas marginalis* (Brown) Stevens 1925 (*Pm*). These bacteria are not only plant pathogens but also saprophytes on plant surfaces and in the soil (Jaques *et al.*, 1995; van Outryve *et al.*, 1989). As symptoms of bacterial softrot have never been observed during the four years of our field experiments, the bacteria apparently survive as saprophytes or in latent infections on the crop. The crop should be colonised before the harvest of the roots, during the root production stage. Harrison (1982) identified rain as a major factor for the spread of softrot bacteria in the field and Elphinstone and Pérombelon (1986) found significant correlations between leaf wetness and the growth of *Ecc* populations on potato leaves.

This paper focuses on irrigation and nitrogen fertilisation of the chicory roots and the influence of environmental factors on the occurrence of softrot bacteria. As nitrogen fertilisation and irrigation of the roots influence both the growth of the chicory plants and the microclimate in the crop, these variables were included in the study.

The variables temperature, rainfall, relative humidity and global radiation at the field site were studied for their influence on the population build-up of softrot bacteria.

Material and Method

Root production in the field - experimental design

A 4-year experiment was conducted at the Experimental Station for Arable Farming in Lelystad, the Netherlands. The experiment was part of a 4-year rotation with winter wheat as the previous crop in 1994-1996 and spring barley in 1997. All 4 experimental fields measured 267m by 21m, with 28 rows of raised beds of 0.75m wide laid out lengthways.
The soil of the experimental fields was a loamy sea-clay with 1.8 % organic matter, 5.8 % Calcium, both measured as percentage dry matter, and a pH of 7.5. The witloof chicory cultivar Rumba was sown in the raised beds in two rows per bed. After emergence, plants were thinned to a within-row spacing of 0.15m, growing a total of 200,000 plants per field. The sowing date depended on the temperature in late May. Sowing proceeded between May 25 and June 5, following a sequence of at least 3 frostfree days. Pre-sowing treatments of the field included a fertilisation with 350 kg Phosphate (P_2O_5) and a herbicide application of 7L carbetamide (300g/L) with 1.5L chlorpropham (400 g/L).

Experimental treatments

Nitrogen and irrigation treatments were applied as experimental factors in the field in a split-plot design. Nitrogen treatment (as NH_4NO_3) consisted of a pre-sowing application of 70 kg/ha and an additional top dressing in June to a total of 140 kg/ha mineral nitrogen in the topsoil (0-0.30m). In August, mineral nitrogen in the topsoil was supplemented to a total of 200 kg/ha nitrogen with an additional top dressing. The control treatment received no additional nitrogen and no supplements. Overhead irrigation was performed from August 18 until harvest in late September. Ten mm water was applied on Mondays, Wednesdays and Fridays when rainfall was under 5 mm during the previous 2-3 days.
For the assignment of the fertilisation and irrigation treatments, the field was divided into 16 plots. The plots measured 10.5 x 30m and consisted of 14 rows, each 30m long. The field was divided crosswise and one half of 8 plots received irrigation, the other half none. In each of these two half fields, 4 plots were randomly assigned to the high nitrogen fertilisation treatment and 4 plots to the control treatment without nitrogen fertilisation. The influence of fertilisation and irrigation on the plants was assessed by measuring the leaf area of ten randomly selected plants per plot in 1994 and 1995.

Measurement of environmental variables

A weather station at approximately 100m distance from the field measured temperature, rainfall, air humidity and radiation continuously. Data were collected from a PT 500 station at a height of 1.5m according to the standard requirements for a meteorological station.

Temperature was measured with a thermocouple in Celsius (°C), relative humidity of the air with a Rotronic electronic sensor (%), global radiation with a Kipp radiometer (W/m^2) and rainfall with a standard rain gauge (mm). All data were measured continuously and electronically stored in a personal computer. Data from the selected months (May through September) were pooled and averaged to obtain monthly averages for the statistical analysis of the experiment.

Collection of bacterial isolates

Ten randomly selected plants from each of the 16 plots were collected each fortnight from May through September. Plants with leaves and roots were used for the assessment of bacterial the number of isolates until July. Thereafter, only the root tops and the leaf base segments of approximately 4cm long were used, having about the same biomass (circa 10 gr/plant) as the entire plants used earlier in the season. The 10 sampled plants or plant parts from each plot were pooled and then divided into two sub-samples of 5 plants or plant parts each. Both sub-samples were shaken with 30-40 ml of 0.1 M sterile phosphate buffered saline (PBS) at pH 6.8 for several hours to release the bacteria from the surface of the plant parts. Two times 25 µl aliquots of the resulting bacterial suspensions were plated on selective media.
Crystal violate pectate (CVP, Cuppels & Kelman, 1974) was used for the detection of *Erwinia* spp. and pseudomonas pectate (PP, Hildebrand, 1971) for the detection of pectolytic *Pseudomonas* spp. Isolation of the pectolytic bacteria was performed as described previously (Schober and Zadoks, 1998). After purification of the pectolytic isolates, the cultures were identified by the Plant Protection Service, Wageningen, the Netherlands, using fatty acid analysis and biochemical tests. The total number of pectolytic isolates was recorded for each plot. The fortnightly data were averaged per month in all four years of the experiment.

Statistical analysis:

The number of pectolytic isolates was $\ln(n + 0.1)$-transformed and used to establish relationships with the environmental variables using linear regression models. Best subset models were determined by stepwise integration of variables into the model. Selection criteria included the Durban-Watson statistic for autocorrelation, sensitivity checks of the independent factors, Mallows' CP , the variance inflation factor (VIF) and analysis of the standardised residuals for autocorrelation. The numbers of isolates and their monthly growth rates were plotted and Pearson's correlation-coefficient (r) was calculated for each environmental variable. Correlations between isolate numbers and environmental factors from each of the four experimental years were tested for significant differences between the years. Data were pooled if no statistically significant differences ($p > 0.05$) between the years were found. Relations between numbers of identified softrot isolates of *Ecc* and *Pm* and environmental variables were analysed using Spearman's rank correlation coefficient.

Results

Effects of irrigation and nitrogen fertilisation

The number of bacterial isolates showed an overall increase throughout the growing season of the witloof chicory crop.

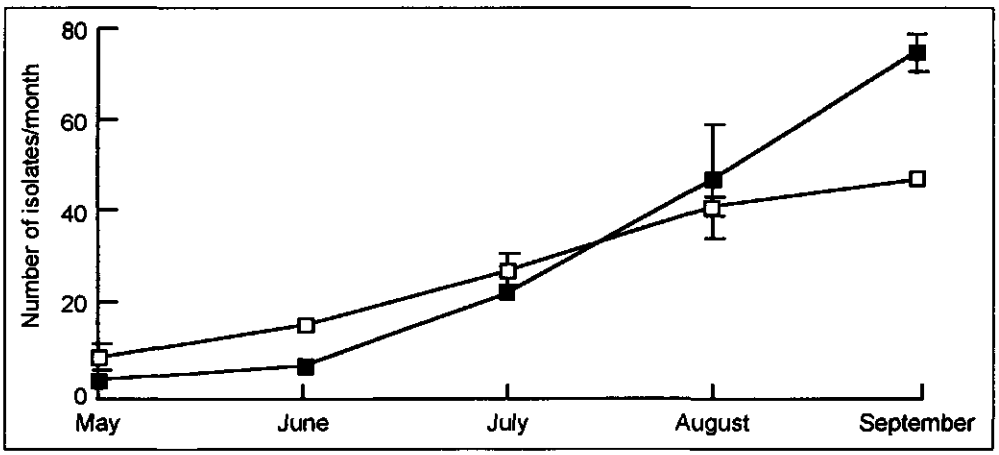

Fig. 1: Monthly numbers of pectolytic isolates on witloof chicory leaves in fertilised (■) and non-fertilised (□) plots in the field during root production. Data are averaged over four years, 1994-1997. Bars represent ± σ.

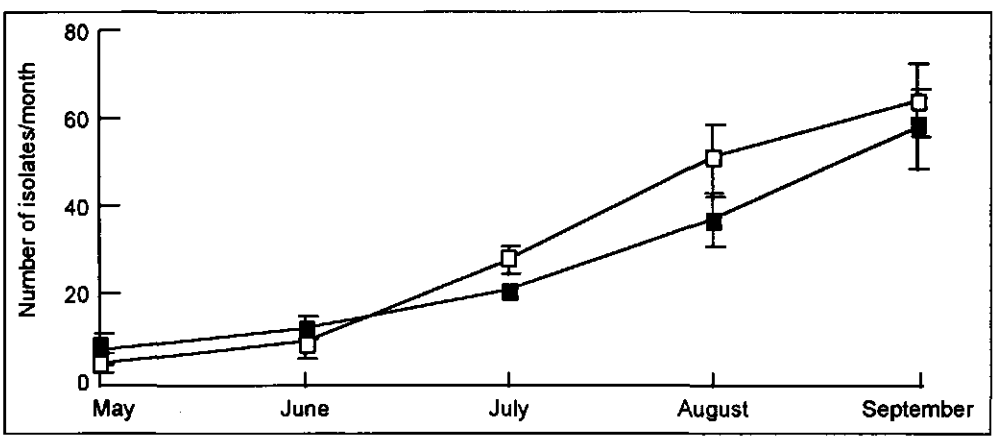

Fig. 2: Monthly numbers of pectolytic isolates on witloof chicory leaves in irrigated (■) and non-irrigated (□) plots in the field during root production. Data are averaged over four years, 1994-1997. Bars represent ± σ.

The monthly average number of isolates increased most strongly in 1996, and was lower but with similar fluctuations in the other three years of the experiment. On average, 0.5 to 2 pectolytic isolates per sample were detected on the leaves of the 10 sampled plants in all four years.

The differences between years were not significant due to the high variance between the plots within years for all sampling dates. Nitrogen fertilisation had a significant effect in September, when more isolates were found in fertilised than in non-fertilised plots (Figure 1). The average leaf area per plant was significantly higher for plants from fertilised plots ($p < 0.01$) in September for both irrigated and non-irrigated treatments. Numbers of isolates increased continuously from May until September. In May and June, at the beginning of the growing season, the total number of isolates was not significantly different for irrigated and not irrigated plots (Figure 2).

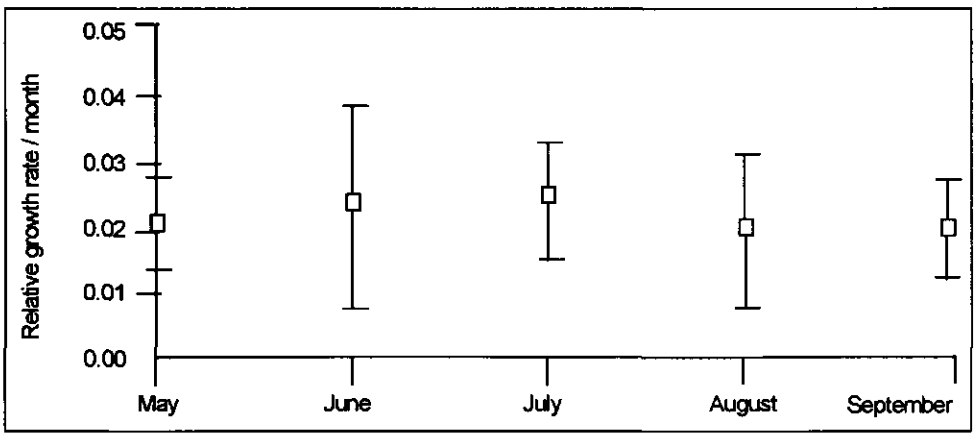

Fig. 3: Relative monthly growth rate of the numbers of pectolytic bacteria isolated from plants during the growing season of witloof chicory roots. Data are averaged over four years, 1994-1997. Bars represent $\pm \sigma$.

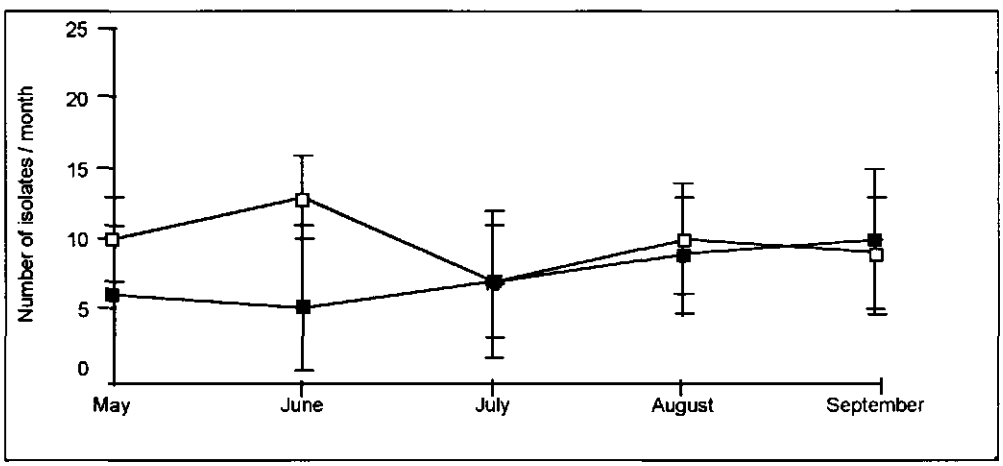

Fig. 4: Monthly averages of numbers of isolates of *Erwinia carotovora* (■) and *Pseudomonas marginalis* (□) isolated from plants during the root production in the field. Data are averaged over four years, 1994-1997. Bars represent $\pm \sigma$.

In July and in August, when irrigation began, the number of bacterial isolates was significantly higher ($p < 0.05$, 51 versus 37 isolates) in non irrigated plots than in irrigated plots.
The relative monthly growth rate, calculated as the natural logarithm of the difference between numbers of isolates of two successive months was greater than zero ($p < 0.001$, Student's one-sample t-test). No statistically significant difference was found among the four years of the experiment ($p = 0.56$, Kruskal-Wallis test statistic). The relative growth rate did not change during the growing season from June until September (Figure 3) and showed no direct relationship with the nitrogen or irrigation treatments. Monthly growth rate averages varied from a minimum of 0.019 in May to a maximum of 0.025 in July. The increase of the number of isolates in May and June was mainly caused by *Pm* and that in August and September by both *Pm* and *Ecc* (Figure 4).

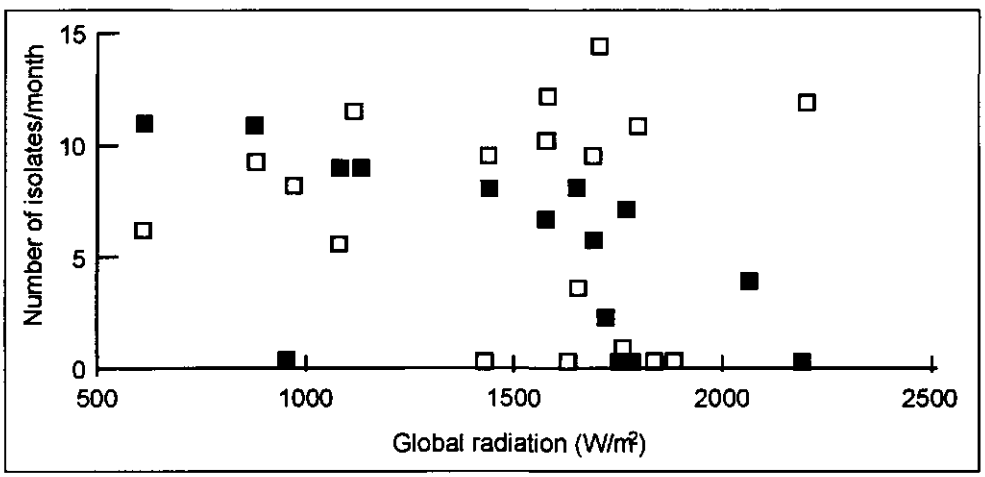

Fig. 5: Numbers of *Erwinia carotovora* (■) and *Pseudomonas marginalis* (□) isolates detected per month at different global radiation levels in the root production field. Data are from 1994-1997.

Influence of environmental factors on the number of isolates of softrot bacteria

Monthly averages for temperature, rainfall and relative humidity did not fluctuate substantially throughout the four growing seasons ($p<0.05$) and these environmental factors did not differ significantly ($p<0.05$) among the four years of the experiment. Monthly averages of temperature and radiation showed a maximum in July, caused by the more extreme conditions in July of 1994 (data not shown). Temperature and radiation averages were significantly higher than in the other months of 1994 ($p=0.05$). Average radiation intensity was lowest in September and average temperature was lowest in May for all four years of the experiment. For the regression model all environmental data were pooled over the four years. Using best subset regression to select an appropriate regression model for the explanation of the monthly numbers of isolates, only the factor time (A, months) contributed significantly to the regression model (Table 1).

The addition of the temperature (B) to the model (model AB) increased the adjusted R^2 from 0.45 to 0.47, decreased the residual sum of squares and provided a better value for Mallows CP.

Although the addition of the temperature term into the regression model (model AB) is not significant ($p = 0.22$), the increase of the adjusted R^2, Mallow's CP and the decrease of the residual sum of squares justified the inclusion of this variable. ANOVA for the regression model AB was significant at $p = 0.002$. The final model AB is $\ln(n+1) = -7.92 + 0.57*T + 0.10*K$ where N is the number of isolates per month per plant(part), T is the time in month (with May = 5) and K is the monthly average temperature in °C. Other combinations of environmental variables in the model made no significant contributions to the model expressed as an increasing adjusted R^2 and decreased the significance of the other variables already in the model. The Durban-Watson test showed the absence of autocorrelation of the random error terms ($p>0.10$) in model AB which supports the inclusion of the variable time in the model. Pearson's correlations of numbers of isolates and environmental factors only showed a strong and positive correlation ($p = 0.001$) with time (months) and an indicative ($p<0.10$) correlation with the average monthly temperature (Table 2).

Characterisation of bacterial isolates

All 291 pectolytic isolates were identified to genus level and 151 isolates were identified as being either *Erwinia carotovora* or *Pseudomonas marginalis*. Analysis of their ratio throughout the growing season showed a predominance of *Pm* early in the season and approximately equal numbers of the two species later in the season and at harvest (Figure 4). The relative decrease of *Pm* isolates from June to July was not followed by a similar decrease of *Ecc* isolates. Environmental factors showed an indicative correlation with the monthly total numbers of both species (Table 3) but only the effect of the global radiation on *Ecc* ($r = -0.69$, $n = 20$, $p = 0.01$) was statistically significant.

The number of *Ecc* isolates declined with increasing radiation levels (Figure 5) and was significantly and negatively correlated with the global radiation (Spearman's rank correlation $r = -0.69$, $n = 20$, $p = 0.012$). The number of *Pm* isolates was not significantly correlated with the radiation levels (Spearman's rank correlation $r = -0.12$, $n = 20$, $p = 0.68$, Figure 5).

Table 1: Unweighted least squares linear regression model (AB) for average number of bacterial isolates ($n = 20$) by time (A) and temperature (B).

Predictor Variables	Coefficient	Standard error	Student's t	p	VIF
Constant	0.52	1.41	-5.60	0.000	
A	0.57	0.16	3.60	0.002	1.1
B	0.10	0.08	1.28	0.216	1.1

Adjusted $R^2 = 0.47$ Residual mean square (MSE) = 0.92

Standard deviation = 0.96

Discussion

This study presents evidence of the environmental dependency of the number of saprophytic isolates of softrot bacteria on witloof chicory leaves and specifically of the influence of two cultural practices, nitrogen fertilisation and irrigation of the crops on the occurrence of softrot bacteria on the witloof chicory plants. Cultural practices influenced the numbers of isolates of softrot bacteria.
The numbers of isolates from the plant samples differed significantly between the irrigated and non-irrigated treatments in August (51 isolates non-irrigated vs. 37 irrigated) and September (64 vs. 58 isolates). This difference between the irrigation treatments levelled off in September, probably due to increasing rainfall that affected both treatments. The sampling interval (two weeks) was supposedly too long to detect population changes caused by rainfall. Possible mechanisms of rainfall-dispersion include splash-dispersal, washing off and forming of aerosols containing bacteria (Goto, 1994). These mechanisms affected the low number of epiphytic or saprophytic populations of softrot bacteria by increasing the numerical variation of the population numbers found in the irrigation treatments.
The development of phytopathogenic species as saprophytes on the surface of witloof chicory leaves and root crowns has consequences for the later stages of the witloof chicory production, the storage and forcing of the roots. As the four growing seasons showed relative uniform weather patterns, which is unusual in the Netherlands, weather data could only be handled at a rather high aggregation level, monthly averages. The number of isolates found on the leaves was significantly and positively correlated with the average monthly temperature. Other environmental factors (rain, air humidity and radiation were negative) gave no significant correlations in the present study.

Table 2: Pearson's correlation-coefficient (r) and their significance (p) for the number of pectolytic isolates (n = 20) on witloof chicory plants and some temporal factors and monthly averages of environmental factors. Data are pooled for four seasons, 1994-1997.

Environmental factor	r	p
Month	**0.70**	**0.001**
Year	0.15	0.53
Rain	-0.12	0.61
RH	-0.06	0.81
Radiation	-0.38	0.10
Temperature	**0.41**	**0.07**

Bold print identifies significant correlation-coefficients.

Identifying the source of epiphytic softrot bacteria, whether migration of growth of resident populations, has been performed by Jaques *et al.* (1995). They showed that in the field the number of isolates of pectolytic bacteria does increase on the leaf surface of broad-leaved endive (*Cichorium endivia* var. *latifolia*) throughout the growing season. He also showed that the size of these populations increased on newly emerging leaves as the plants aged, but he was unable to confirm the point using strains artificially applied on the plants at the beginning of the growing season.

He concluded that environmental conditions and the accessibility of leaves to migrating airborne or splashborne bacteria are major factors in the regulation of the population dynamics of pectolytic bacteria in the field.
According to Vantomme *et al.* (1989) approximately 14% of all bacterial strains found on leaves of witloof chicory plants in the field are pectolytic strains of the genera *Erwinia* and *Pseudomonas*. Most of the isolates of pectolytic bacteria can be found on the leaf base and near the stem in close proximity to the soil (Tsuyama, 1974). According to Tsuyama's study, leaf age and environmental conditions near the soil surface under the canopy explain the difference in numbers of pectolytic bacteria between lower and upper plant parts.
In our study, the general trend was an increase of the number of isolates throughout the growing season at a constant and positive growth rate, indicating an increase of the numbers of populations or the numbers of bacteria within the populations.
The influx of pectolytic bacteria due to migration might be responsible for the early seasonal increase in May and June observed in all four years of the experiment. The relative importance of the bacterial fluxes, however, could not be detected in our study because they are below the detection-level of the techniques used (circa 10^4 cfu/ml).
A survey by De Boer (1983) of the presence of *E. carotovora* on leaves of potato crops in the Pemberton Valley (Canada) using similar detection techniques showed a similar population build-up on potato leaves at one farm location and almost no bacteria at another location. At both locations the number of *E. carotovora* increased rapidly when susceptible tissue in the form of vine debris became available and the bacteria switched from their saprophytic existence on the leaves to a pathogenic existence in the plant tissue. Witloof chicory leaves also ripen and decay at the end of the growing season, suggesting a similar switch in the behaviour of the bacteria. This hypothesis is supported by the occasional occurrence of diseased plants in witloof chicory fields (but not in our field), caused by *Pm* and known as leaf necrosis. This observation points to a change in relative importance of migration and multiplication of the bacteria during the growing season.
The ratio of isolates belonging to the genera *Pseudomonas / Erwinia* shifted towards 1 at harvest time in the favour of the *Erwinia's*. As the canopy of the crop did not close before August, the sensitivity of the *Erwinia's* to high global radiation could be the factor inhibiting their growth in the early season. Ranganna *et al.* (1997) reported the total inhibition of softrot development in potatoes at UV-radiation levels of 15 kJ/m^2.

Table 3: Spearman's rank correlation coefficients (r) and their significance (p) for the number of *Ecc* and *Pm* -isolates (n = 20) and some monthly averages of environmental factors. Data pooled for four seasons, 1994-1997.

	Ecc		*Pm*	
Environmental factor	r	p	r	p
Rain	0.11	0.77	0.63	0.25
RH	-0.03	0.92	0.39	0.17
Radiation	**-0.69**	**0.01**	-0.12	0.68
Temperature	0.18	0.57	-0.10	0.74

Bold print identifies significant correlation-coefficients.

The resulting inhibition of the growth of *Ecc* in the field reduced the competition with *Pm*, allowing the latter to take advantage of the free niches resulting in the numerical predominance of *Pm* from May until August. After closure of the canopy, the water availability in the microclimate under and in the canopy differed from that at the top of the canopy. The change in the microclimate supposedly had a significant effect on bacterial numbers (Kinkel, 1997; Hirano *et al.*, 1995) and resulted in the increase of *Ecc*.
The general pattern of the population changes as observed in this study was also found on broad-leaved endive studied by Jaques *et al.* (1995). The plants acquired a microbial community early in the season and the plants kept their microbial communities although populations may have fluctuated during the season. Apparently, competition and environmental factors were more important than influx from external sources once the bacterial populations had established on the leaves. The role of competition emphasises the importance of the saprophytic capabilities of the softrot bacteria *Ecc* and *Pm*. Further analysis of the softrot severity during the forcing stage will show whether the increased number of pectolytic isolates in the field has any influence on the disease severity during forcing of the witloof chicory heads.

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Chapter 6

Survival of softrot bacteria during the storage of witloof chicory roots

Survival of softrot bacteria during the storage of witloof chicory roots

Schober, BM and Zadoks, JC.

Summary

During the cold storage of witloof chicory roots, changes in the populations of the softrot bacteria *Erwinia carotovora* subsp. *carotovora* (*Ecc*), *Erwinia carotovora* subsp. *atroseptica* (*Eca*) and *Pseudomonas marginalis* (*Pm*) were investigated from October (directly after harvest) until March of the next year. Colonisation incidence of *Ecc* and *Pm* changed during the early weeks of storage in each of the 3 years of the experiment and significantly increased after December, approximately 6-8 weeks after the roots had been placed in storage. After inoculation, in the storage of 1996, populations of *Eca* decreased continuously from $5.8*10^5$ cfu ml^{-1} to 10^3 cfu ml^{-1} four weeks later in mid-November without any further statistically significant increase during the rest of the storage period. Timecourse analysis of the monthly colonisation incidence of *Ecc* and *Pm* over the three years revealed a significant dependency of the incidence on the incidence two weeks earlier. Also, high colonisation incidences during storage coincided with high disease incidence during the following forcing period of the roots. Therefore, a forecast of disease incidence during forcing of the witloof chicory heads may be envisaged.

Introduction

Witloof chicory (*Cichorium intybus* L. var. *foliosum* Hegi) is an important vegetable in Belgium, France and the Netherlands. The edible product, the witloof chicory heads, consists of the etiolated leaves of the biennial plant grown from the roots in the dark under moist conditions. The production process is called forcing of the roots. Witloof chicory plants are sown for root production in May and their growing season lasts until the period of September to November, depending on the cultivar. After harvest, the roots are placed in cold storage for vernalisation from 4 weeks up to 10 months until forcing of the roots.

The introduction of the hydroponics system for forcing, which reduces forcing time to three weeks, has increased colonisation incidence on the witloof chicory heads and altered the composition of the bacterial flora (Vantomme *et al.*, 1989).

Healthy roots can develop a slimy softrot of the growing heads during the last days of forcing which may induce losses up to 60% of the total crop. This softrot of the heads is caused by several types of phytopathogenic bacteria, of which *Erwinia carotovora* subsp. *carotovora* (Jones) Bergey *et al.*. 1923 (*Ecc*) and *Pseudomonas marginalis* (Brown) Stevens 1925 (*Pm*) are the most common in the Netherlands. In addition, *Erwinia carotovora* subsp. *atroseptica* (van Hall) Dye 1969 (*Eca*) is a major softrot pathogen of witloof chicory in France and Belgium (Vantomme *et al.*, 1989).

In the field, the softrot bacteria are present on the roots (van Outryve, 1988) and the leaves (van Outryve, 1989) of healthy witloof chicory plants.

After harvest of the roots, the bacteria are carried along into storage on roots and leaf stubs. As nitrogen fertilisation of the roots influence both the growth of the chicory plants and the microclimate in the crop, this variable was included in the study.

Studies on the storage of potato tubers (Pérombelon & Lowe, 1975) show that the numbers of the softrot bacterium *Ecc* will decline under storage conditions comparable to those of witloof chicory roots. Anaerobic conditions, an increasing metabolic activity of the potato tubers during late storage and fluctuating storage temperatures induce cell leakage into the intercellular spaces of the tuber's tissue. The leaked cell contents are utilised by the softrot bacteria and allow rapid growth of the bacteria in the intercellular spaces of the plant tissue (Pringle *et al.*., 1991). These studies suggest the storage conditions of the witloof chicory roots to be a key element in the development of bacterial softrot during forcing.
Witloof chicory roots from commercial farms are stored in wooden boxes of 1 - 2.5 m^3 which contain 300-500 kg of roots. The boxes are kept at low temperature and controlled humidity in storage cells to provide optimal storage conditions for the roots during vernalisation. As indicated by the studies on potato tubers, these conditions may not be sufficient to control the number of bacteria present on the roots.
The aim of this study was to monitor changes in bacterial populations on witloof chicory roots held under commercially applied storage conditions and to assess the development of bacterial softrot during forcing as a result of the presence of softrot bacteria on the witloof chicory roots in storage.

Materials and methods

Root storage

Witloof chicory roots cv. Rumba were grown in three years (1994-1996) on raised beds at the Experimental Station for Arable Farming (PAV) in Lelystad, the Netherlands. Two nitrogen fertilisation regimes were established according to current cultural practices in the Netherlands, representing the upper and lower limits of nitrogen applications in witloof chicory root production. Two treatment levels were used, in one a total of 200 kg nitrogen was applied in two applications during the growing season from May until September (high nitrogen treatment) and in the other no nitrogen fertilisation was applied (low nitrogen treatment) as described previously (Schober and Zadoks, accepted).
Roots from plots with the high and low nitrogen treatments were harvested separately in late September by mechanically lifting and defoliating the plants. Defoliation resulted in a leaf stub of 5 cm for the protection of the apical growing bud during handling and storage of the roots. Roots were mechanically collected and packed in wooden storage bins of 1.5 m^3, routinely treated against *Sclerotinia sclerotiorum* with Ronilan™ by immersion of the bins and then pre-cooled in a climatised storage room at a temperature of 0.5° - 0 °C. After 4 weeks of pre-cooling, roots were packed into bins containing 70-100 roots each, the bins were piled 6-8 bins high and placed in a temperature controlled storage room at 0.5 °C under a loose cover of perforated plastic foil to reduce evaporation.
In total, approximately 30,000 roots were stored in circa 300 bins from October until March in each of the three years. Temperature and humidity in the storage cells were computer controlled with a tolerance of 0.2 ° C for temperature and of 5% for relative humidity.

Roots were kept moist to counteract evaporation by spraying the piles of bins with cold tap water once every month, temporarily removing the plastic cover. In 1996, two moisture regimes were established during the storage of the roots: wet storage by placing plastic foil around each bin separately (wet treatment) and dry storage by covering the piles loosely with perforated plastic foil (dry treatment) and further treated in the same way as in the two previous years.

Sampling roots for softrot bacteria

To sample the roots for the presence of softrot bacteria, 8 bins containing non-fertilised roots and 8 bins with fertilised roots were randomly selected at the beginning of a storage period, stacked separately from the other bins in the same storage cell, treated in the same way as the other bins and used throughout the entire sampling period. In 1996, two humidity treatments during storage were investigated and roots were sampled from 16 bins in two separate piles of 8 bins placed in the same storage cell.
Samples were drawn every two weeks during the storage season, two roots were taken from each bin (the basic statistical sampling unit) to a total of 16 roots per nitrogen treatment with a total of N = 32 roots per sampling date. In 1996, the 16 roots per nitrogen treatment were sampled from both humidity treatments with n = 32 roots per humidity treatment and a total of N = 64 roots per sampling date.
The leaf stubs and the apical growing bud (the root top) from each root were examined for the presence of softrot bacteria. The leaf stubs and growing bud were separated from the roots, cut fine and washed in 15 ml 0.1M phosphate buffered saline (PBS), pH 6.8. The suspensions from each root were shaken for 2 hrs to release micro-organisms. Samples of 25 µl of the suspension were plated on selective media. Crystal violate pectate (CVP, Cuppels & Kelman, 1974) was used for the detection of *Erwinia* spp. and pseudomonas pectate (PP, Hildebrand, 1971) for the detection of pectolytic pseudomonads. To obtain isolated colonies, the sample of the bacterial suspension was spread on water agar (Oxoid) using a sterile glass rod. Subsequently, the remaining bacteria still present on the rod were streaked on one plate of CVP agar and then one plate of PP agar. Bacterial colonies developing distinct cavities in the selective media were re-isolated on Tryptic Soy Agar (Oxoid, TSA) and King's B (Oxoid).
Pure cultures of isolates reacting positively on the selective media were used in a pathogenicity test on witloof chicory leaves from freshly harvested witloof chicory heads of cultivar Rumba. Bacteria from a single colony were picked up with the spatula of an inoculation needle and scratch-inoculated to surface-disinfected leaf parts (circa 4 cm long and wide) placed in a 45 mm Petri dish to induce tissue wounding. Wound-inoculated leaves in closed Petri dishes were placed in plastic boxes on filter paper moistened with demi water containing 0.3 mM NaN_3 to prevent spreading of the bacteria. Boxes were closed with a lid, sealed for high humidity and incubated for 2-5 days at 2 7°C. Isolates were classified as softrot positive if the inoculated leaf part showed distinct softrot after the incubation period. The softrot positive isolates were identified as to sub-species level by the Plant Protection Service, Wageningen, The Netherlands.

Disease incidence during forcing of the witloof chicory heads: Witloof chicory heads were forced from roots three times per storage period, in October, December and March. Roots from the different humidity and fertilisation treatments were forced separately. After forcing of the roots the witloof chicory heads were visually assessed for symptoms of bacterial softrot during harvest. Distinct maceration of the witloof chicory tissue of the head was the criterion for disease assessment. Samples from softrot positive witloof chicory heads were routinely checked for the presence of softrot bacteria and isolates were characterised as described above and identified by the Plant Protection Service.

Monitoring population densities of *Eca*

In 1996, 200 roots were artificially inoculated before storage with a suspension of *Eca* (type-strain *Erwinia carotovora* subsp. *atroseptica* IPO-161 from the culture collection of the IPO-DLO (DLO - Research Institute for Plant Protection, the Netherlands)) in Nutrient Broth (Oxoid) using a spray dispenser. The suspension had a density of approximately 10^9 *cfu* ml^{-1}. *Eca* was chosen as a softrot pathogen to compare results with earlier work (Vantomme *et al.*, 1989) from France and Belgium on witloof chicory using this pathogen. Roots were equally sprayed with the suspension, applying 1 ml on each root. The inoculated roots were stored in two separate bins for the two humidity treatments. Two roots were taken from each bin of the two treatments every fortnight to sample for *Eca*.
The root tops were sampled for the presence of *Eca* using sampling techniques as described above and quantitative immunodetection by spiralplating and immunofluorescence colony staining (IFC; Schober & Van Vuurde, 1997) to quantify the numbers of *Eca* present in the sample. As a positive control, suspensions of a 24-48 hrs old culture on Nutrient agar (Oxoid) of the same strain of *Eca* were used for immunodetection at a density of approximately 10^4 cfu ml^{-1} in 0.01M sterile PBS at pH 6.8.

Statistical Analysis

The number of roots per sample of 16 roots per treatment from which softrot bacteria were isolated was defined as the colonisation incidence. The fraction (from 0 to 1) of roots with *Ecc* and the fraction of roots with *Pm* were added, so that the colonisation index for softrot bacteria may run from 0 to 2. Colonisation incidence was $\sqrt{}$-transformed and analysed by ANOVA (Gomez *et al.*., 1985) to test for differences between treatments at each sampling date and to test for timecourse variation within sampling years. Comparison of treatment averages and timecourses were performed using Tukey's HSD for means at $\alpha = 0.05$. Data on the number of *Eca* during storage in 1996 were log-transformed for analysis.
Tests for autocorrelation were performed using the data of the three sampling periods per year as one continuous set. The autocorrelation function (acf) and the partial autocorrelation function (pcf) were calculated for the dataset to analyse timecourse variation for the two-weekly lags (acf) and to study correlations between different time lags after removal of all intervening lags (partial autocorrelation function, pcf). Calculations were performed using the statistical software SPSS™ and Statistix™.

Results

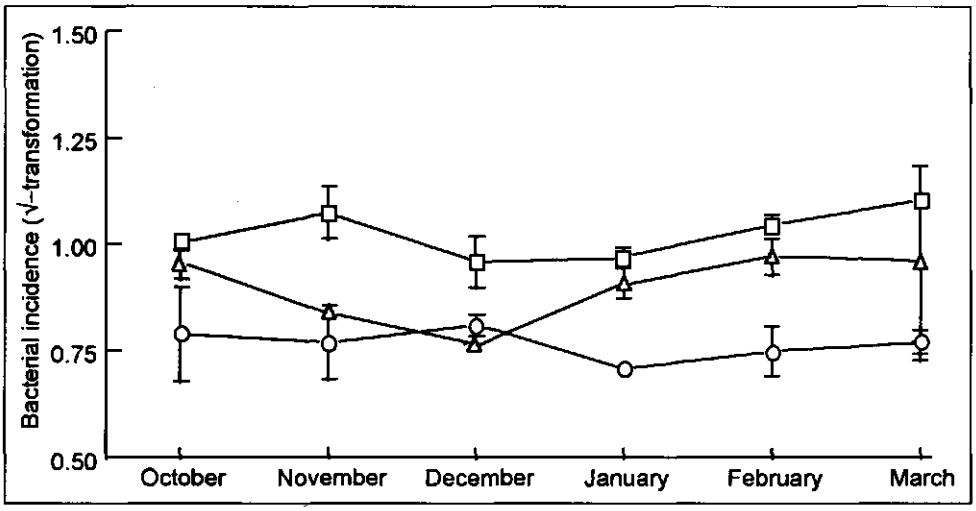

Fig. 1: Development of colonisation incidence of the softrot bacteria *Ecc* and *Pm* during storage of witloof chicory roots from October until March in three consecutive years (O 1994, Δ 1995 and □ 1996). Colonisation incidence is measured as the sum of the fractions of roots with *Ecc* and *Pm*.

Colonisation incidence of softrot bacteria during storage

The colonisation incidence of *Ecc* and *Pm* on witloof chicory roots changed during storage and varied between and within years (Figure 1).
Colonisation incidence was highest in 1996, lowest in 1994 with 1995 intermediate. Low values of the colonisation incidence were found in December (1995, 1996) and January (1994, 1996). Later during storage the colonisation incidence increased, strongly in 1995 and 1996 and less in 1994. Annual mean colonisation incidence significantly (Tukey's HSD = 0.156) increased from 0.09 in 1994 to 0.33 in 1995 and 0.56 in 1996. Mean annual colonisation incidence increased more clearly for the low N treatment than for the high treatment. The increases roughly paralleled that of the disease incidence of witloof chicory heads after forcing of the roots (Figure 1). In the first two months of the storage period, 63-67% of all isolates of softrot bacteria were identified as *Pm*, and the others as *Ecc*. The ratio changed in favour of *Ecc*; from December until February 87-98% of all isolates were identified as *Ecc*. After February, the ratio changed again and at the end of the storage period isolates of both pathogens were found in about equal numbers.

Disease incidence during forcing after storage of the roots

High colonisation incidence during storage in 1996 coincided with statistically significant higher disease incidence during forcing of the witloof chicory heads in all three forcing periods of 1996 (Table 1). In a similar way, the low colonisation incidence in late storage in 1994 coincided with a significantly lower disease incidence during late forcing in March 1994 compared to the forcing in October and January.

The annual average disease incidences after forcing of roots from low and high nitrogen treatments differed significantly in 1994 (Tukey's HSD = 0.04) and 1995 (Tukey's HSD = 0.05) but not in 1996 (Tukey's HSD = 0.08). In general, high nitrogen treatment during root production led to high disease incidence during forcing. This was true for all three forcing seasons in 1994 and 1995 and for the forcing seasons in October and March of 1996.

Temporal analysis of colonisation incidence during storage of the roots

The temporal autocorrelation of the colonisation incidence over three years showed a significant correlation for lag 1: $r_1 = 0.55$, exceeds the confidence limit of two standard errors (2 * s.e. = ± 0.33), indicating the dependency of the observation on the observation one sampling period, i.e. two weeks, earlier.
This result suggests that there may be an autoregressive AR (1) component in the observations. The partial autocorrelation function (acf) had a significant value at lag 1: $r_1 = 0.55$ (with 2 * s.e. = ± 0.35), which also supports the idea of an time-dependent component in the data.

Occurrence of softrot bacteria during 1996 with controlled humidity in storage

The two humidity regimes of 1996 produced small but statistically significant differences in temperature and relative humidity measured in the storage bins (data not shown). The monthly averages (n = 16) for the four treatment combinations varied considerably, up to a fourfold, but in an irregular pattern (Table 2). Averages over time (n = 32) and nitrogen treatments (n = 160) varied less and differences were generally not significant. Only in October, averages between nitrogen treatments differed significantly in both the dry and the wet humidity treatment.

Table 1: Average disease incidence per treatment combination and its standard error during forcing in 1994 - 1996 with two nitrogen treatments applied during the root production. HSD between fertilisation treatments = 0.02 ($\alpha = 0.05$).

	Average ± s.e. of disease incidence		
Year	Forcing season	Nitrogen Low	High
1994	October	0.048 ± 0.09	0.138 ± 0.13
	December	0.004 ± 0.01	0.149 ± 0.15
	March	0.003 ± 0.01	0.037 ± 0.05
1995	October	0.029 ± 0.02	0.109 ± 0.13
	December	0.126 ± 0.11	0.219 ± 0.11
	March	0.102 ± 0.11	0.070 ± 0.09
1996	October	0.455 ± 0.14	0.351 ± 0.14
	December	0.126 ± 0.11	0.119 ± 0.13
	March	0.110 ± 0.05	0.185 ± 0.10
	Average	0.113 ± 0.16	0.158 ± 0.15

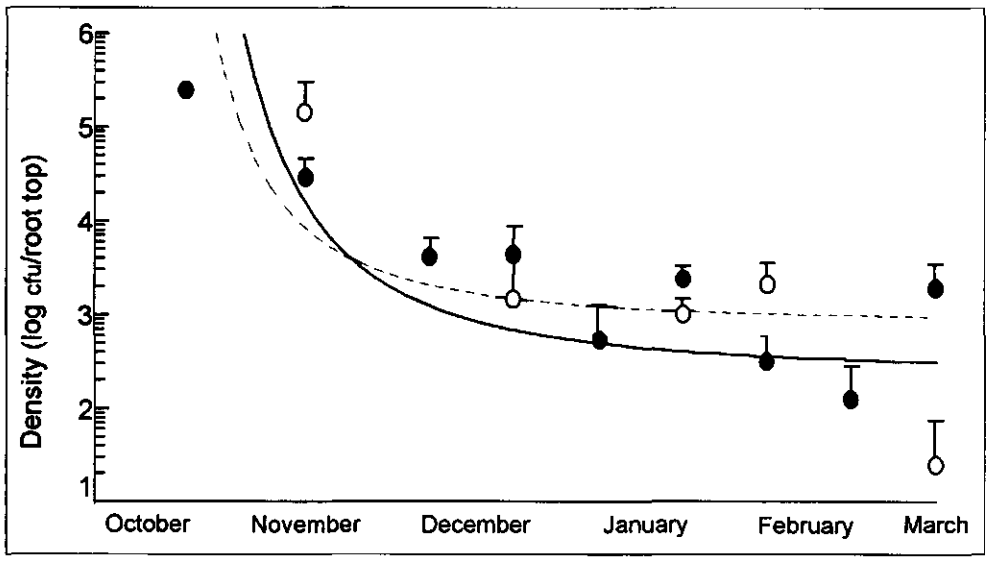

Fig. 2: Population development of *Eca* after artificial inoculation of witloof chicory roots in October at the beginning of the storage period and monitored from October until March under dry (O, dashed line) and wet (●, solid line) storage conditions of the roots in 1996.

Development of populations of *Eca* on roots in 1996:

Population development of *Eca* during storage of roots followed an approximately exponential decline (Figure 2). Significantly lower numbers of *Eca* were found after storage periods of 5 weeks and longer ($4.3*10^3$ - 100 cfu ml^{-1}) compared to 0-2 weeks of storage ($2.3*10^4$ cfu ml^{-1}).
The increase of the colonisation incidence during storage from January until March, caused by *Ecc* and *Pm*, coincided with a similar although not statistically significant increase of the numbers of artificially inoculated *Eca* in January and February.

Table 2: Colonisation incidence of witloof chicory roots and its standard error during storage for two humidity treatments in 1996 with two nitrogen treatments applied during root production. HSD for nitrogen treatments = 0.22 for dry humidity treatment and HSD = 0.24 for wet humidity treatment (α=0.05).

	Relative humidity in storage			
	Dry		Wet	
	Nitrogen fertilisation		Nitrogen fertilisation	
Date	High	Low	High	Low
October	0.44 ± 0.04	0.69 ± 0.04	0.56 ± 0.13	0.19 ± 0.04
November[a]	0.63	0.56	0.56	0.63
December	0.44 ± 0.13	0.38± 0.18	0.38 ± 0.09	0.50 ± 0.09
January	0.69 ± 0.22	0.31 ± 0.13	0.56 ± 0.04	0.19 ± 0.04
February	0.56 ± 0.13	0.50 ± 0.09	0.56 ± 0.04	0.75 ± 0.09
March[a]	0.19	0.31	0.56	0.38

[a] Only one assessment

Decline of *Eca* during storage was exponential for both humidity treatments and in December and March significantly less *Eca* were found in the dry humidity treatment than in the wet humidity treatment (Table 3).

Discussion

The colonisation of stored witloof chicory roots with softrot bacteria attained its minimum in December of all three years of the experiment and increased thereafter in two out of three years (Figure 1). Identification of the isolates showed that both *Ecc* and *Pm* contributed to this increase. These results are in agreement with results obtained by Pringle *et al.*. (1991). Although Pringle performed his experiments with potato tubers, it seems that the mechanisms involved in the population changes are comparable.

Both experiments were performed with fleshy storage organs containing high concentrations of sugars (both lower and higher) and storage conditions of these tubers were comparable, although higher temperatures were applied in potato storage. Pringle mentioned an increasing metabolic activity (sprouting) of the potatoes as a major factor for the increase in bacterial populations. The one potato lot which did not show this bacterial increase, did not sprout. In witloof chicory, new metabolic activity of the stored roots is observed after December as growth of the apical bud and formation of new leaves.

The crucial role of oxygen is emphasised by Pérombelon & Lowe (1975). Free water, caused by condensation on the surface of potato tubers, created local pockets of anaerobic conditions during storage where *Ecc* can multiply and cause disease. In addition, the anaerobic conditions can cause membrane instability in the tuber tissue leading to cell leakage. The leaked cell contents allow rapid growth of the bacteria. Water sprays applied during witloof chicory root storage supposedly create similar pockets of anaerobic conditions, enhancing the decomposition of the leaf stubs by common anaerobic bacteria such as *Erwinia* sp., *Clavibacter* sp. and *Bacillus* sp. (Goto, 1992). The general decay of leaf stubs by several micro-organisms including fungi, emphasises another aspect of population changes of the softrot bacteria during storage.

Table 3: Development of *Eca* on witloof chicory roots during storage for two different humidity treatments in 1996. Data are log-transformed numbers of cfu ml^{-1} . HSD = 0.86 between humidity treatments and HSD = 1.10 for timecourse intervals (α = 0.05).

	Relative humidity in storage		
Time	Dry	Wet	Average
October	4.3 ± 1.19	4.39 ± 0.36	4.37 ± 0.85
November	3.6 ± 0.07	3.60 ± 0.18	3.63 ± 0.13
December	0.4 ± 1.30	1.63 ± 1.77	1.04 ± 1.61
January	3.0 ± 0.16	3.39 ± 0.12	3.20 ± 0.25
February	3.2 ± 0.29	2.38 ± 0.38	2.83 ± 0.58
March	0.0 ± 0.00	1.74 ± 1.18	0.87 ± 1.21

Saprophytic decomposition of the leaf stubs always involves fluorescent pseudomonads, the most common soil-borne bacterial species (Klement *et al.*., 1990). Strictly aerobic, they will persist during storage due to their strong saprophytic capabilities in aerobic spaces on the roots. The increasing populations of the aerobic species *Pm* suggest that they can initiate softrot during forcing when present near wounded tissue of the growing witloof chicory head.
Due to its stronger pathogenicity, expressed as the amount of pectic enzymes produced (Knösel & Lange, 1979), *Ecc* will supposedly outnumber *Pm* during the development of softrot and thus will be the most numerous causal organism in the advanced stages of the disease during forcing. In healthy witloof chicory heads after forcing, 35% of all the bacteria appear to be pectolytic pseudomonads (Van Outryve *et al.*., 1989) and from diseased witloof chicory heads with softrot from our experiments 40% and 98% of the identified softrot isolates from diseased witloof chicory heads appeared to be *Ecc*.
Population changes of *Eca* differed from those of *Ecc* and *Pm*. Initial numbers of *Eca*, obtained by artificial infection, decreased rapidly during the first weeks of storage and did not increase significantly later in the storage period (Figure 2). Similarly, Van Vuurde & de Vries (1994) found a continuous decrease of *Eca* populations on the surface of potato tubers during storage. Apparently, *Eca* has other growth properties than *Ecc* under the storage conditions typical for harvested potatoes and witloof chicory roots. Under in vitro conditions, *Eca* has a lower absolute growth rate than *Ecc*, making it impossible for *Eca* to outgrow *Ecc* (Pérombelon & Salmond, 1995). Also, *Eca* is known to be sensitive to competition by other Erwinia spp (Jones *et al.*., 1994). As witloof chicory roots harbour a variety of bacterial species (Van Outryve, 1988) *Eca* probably cannot compete on the surface of the roots and leaf stubs so that its population will decrease below the detection level. Nevertheless, research on bacterial softrot in Belgium and France reported *Eca* as the major causal organism of softrot in witloof chicory (Vantomme *et al.*., 1985 and 1989; Sarrazijn & Bockstaele, 1989; Gallois *et al.*., 1992) though results in these reports indicated that the causal organisms can be described as a separate cluster of isolates, clearly distinct from typical potato strains of *Eca*.
The colonisation incidence of the softrot bacteria rose each year due to a build-up of softrot bacteria at the field site. Analysis of the occurrence of softrot bacteria during the root production in the field showed a similar rise of the colonisation incidence (Schober & Zadoks, submitted), especially during August and September, the last two month of the root production prior to harvest. An increasing disease incidence during forcing of the roots in October and December was also found during this experiments. This evidence suggests that a population build-up in the field can cause higher disease incidence during forcing up to 5 months later. The observed decline during storage is not strong enough to reduce the numbers of bacteria carried along on the roots. Time series analysis might eventually lead to a forecast of late disease incidence based on an early assessment of the colonisation incidence during root storage. First results are promising but the time limit of the prediction has yet to be established. Forecasting disease incidence could provide a witloof chicory grower with the means to assess the risk of bacterial softrot during forcing. In addition, a model for forecasting bacterial softrot should incorporate environmental factors such as temperature and humidity since these are known to affect the development of bacterial populations during storage of witloof chicory roots.

Acknowledgements

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Chapter 7

Incidence and distribution of bacterial softrot during forcing of witloof chicory

Incidence and distribution of bacterial softrot during forcing of witloof chicory

Schober, BM and Zadoks, JC

Summary

Witloof chicory can develop a slimy softrot, which may induce losses up to 60% of the crop. The influence of cultural practices on disease incidence and its distribution and aggregation was studied in a three years experiment at two forcing temperatures. Mean disease incidence, expressed as proportion of chicory heads with softrot, was lowest in 1994 and highest in 1996 and ranged from 0.07 - 0.13 in the early forcing season to 0.15 - 0.18 in the two later seasons. Chicory heads forced at low temperatures and grown from roots receiving low nitrogen fertilisation or immersed in 3% calciumchloride had a relatively low disease incidence. Chi-square analysis for goodness of fit for the beta-binomial distribution for disease incidence data was highly significant with $\theta = 0.15$ ($n = 136$, $p = 0.003$) for high temperature and $\theta = 0.11$ ($n = 136$, $p = 0.016$) for low temperature treatment during forcing. Calciumchloride application as a root immersion treatment and low nitrogen levels during root production decreased the disease incidence and increased the aggregation parameter θ. The combination of decreased disease incidence and increased aggregation is explained by small but discrete foci of diseased chicory heads in the forcing trays.

Introduction

Witloof chicory (Cichorium intybus L. var. foliosum Hegi) is an important vegetable in Belgium, France and the Netherlands. The edible product, the chicory heads, consists of the etiolated leaves of the plant grown on the roots in the dark under moist conditions. The production process is called forcing of the roots.

The introduction of the hydroponics system for forcing, which reduced forcing time to three weeks, increased disease incidence on the chicory heads and altered the composition of the bacterial flora (Vantomme *et al.*, 1989). Apparently healthy roots can develop a slimy softrot of the growing heads during the last days of forcing which may induce losses up to 60% of the total crop. This softrot of the heads is caused by several phytopathogenic bacteria, of which *Erwinia carotovora* subsp. *carotovora* (Jones) Bergey *et al.* 1923 (*Ecc*) and *Pseudomonas marginalis* (Brown) Stevens 1925 (*Pm*) are the most common in the Netherlands.

Although studies (Schober & Zadoks, 1998) have shown that the pathogens are introduced into the forcing cells on the roots and leaf stubs, little is known about the early pathogenesis during forcing and the spatial distribution of the disease in the forcing trays.

As chicory heads can be contaminated with softrot bacteria long before disease develops, the spatial distribution of the disease can help to gain insight in its post harvest pathology, when apparently healthy chicory heads develop softrot in the marketing stage. The symptoms of bacterial softrot appear only at the end of the forcing stage (Van Saane & Van Kruistum, 1987), when the chicory heads are fully grown and densely packed in the forcing trays. The economically optimal packing of the roots leads to a very dense packing of the chicory heads at harvest time, the aim being to obtain as big heads as possible without damage caused by the dense packing. Spread of softrot occurs from one chicory head to another, generally causing foci of disease. Leaking nutrient solution of the hydroponic system along the stacked forcing trays as well as high relative humidity create a water film on the crop which allows the bacteria to move and to reach new places of infection.
Cultural practices such as a modest nitrogen fertilisation during chicory root production (Van Saane & Van Kruistum, 1987) given as soil fertilisation and top dressings, application of calciumchloride using root- immersion after harvest (Jolivet *et al.*, 1988) and stringent environmental control during forcing (Van Kruistum, 1997) have been shown to reduce disease incidence.
The aim of this study was to investigate the effects of these cultural practices on softrot development during the forcing of a susceptible cultivar and to study their effects on the spatial distribution of bacterial softrot.

Material and methods

A three years experiment was conducted at the Experimental Station for Arable Farming in Lelystad, the Netherlands. Chicory production was performed according to standard practice (Van Kruistum, 1997). It involved the production of chicory roots in the field, their cold storage for vernalisation during at least four weeks and the forcing of the chicory heads in forcing chambers on a hydroponics system at two temperature regimes. Bacterial softrot develops during the last days of the forcing period only.

Root production and storage

Chicory roots were grown following standard agricultural practice and harvested in September. Nitrogen fertilisation of the roots during the growing season was applied to 8 randomly chosen plots out of 16 plots of the root production field in two applications to a total of 200 kg nitrogen (high nitrogen treatment). The remaining 8 plots received no additional nitrogen treatment (low nitrogen treatment). calciumchloride was applied as an experimental factor before storage (low and high calcium). 50% of all roots per plot were immersed in their storage bins in a 30g/l solution of calciumchloride (CaCl2) for three minutes, the remaining roots were untreated. All roots were stored in bins containing 200 roots each in a climatised storage room at a temperature of 0 - 0.5°C until forcing.

Forcing of the roots

Forcing was performed three times a year, in October, December and March using four temperature-controlled forcing cells each time. Forcing lasted 20-23 days. The temperatures of the air and the processing water were used as an experimental factor. Two forcing cells were set at low temperature (low) and two cells at high temperature (high).

The water and air temperature of the cells varied with the forcing season to optimise head growth and quality according to standard practice (Van Kruistum, 1997). For "high" it ranged from 21°C water/18°C air in October and December to 18°C water/15°C air in March. For "low" it ranged from 18°C water/15°C air in October and December to 15°C water/12°C air in March.
Roots were placed in forcing trays, up to 350 roots per tray, with 8 trays per forcing cell. One tray contained only roots from one combination of nitrogen and calcium treatments. The four treatment combinations, each in two replicates per cell, were placed in four stacks of two trays. Each stack, with two replicates, was connected to a recirculation unit of the nutrient solution. In each forcing cell, the four stacks of two trays were connected to four separate hydroponics systems. Each unit of the hydroponics system was computer controlled and the nutrient solution was held at a constant EC of 2 mS using a continuous supply of nutrients during the forcing period. The trays, with variable numbers of forced chicory heads (200-350), were used as the basic sampling units.

Statistical analysis

The number of diseased chicory heads per tray was counted at harvest of the heads. The fraction of diseased heads per tray was called the disease incidence. The number of healthy chicory heads per tray was counted and all visually healthy heads with a length – diameter ratio of 3 or higher were classified as quality class 1.

Beta-binomial distribution

The delays between treatments (nitrogen fertilisation and calcium application) and response (disease incidence) varied from one to ten months according to the combination of treatments and the forcing period. The delayed response caused considerable variation of disease incidence although the experiments were performed under highly conditioned circumstances. Standard statistical analysis was not feasible as the treatment effects were only significant at a high aggregation level of the disease incidence data.
The use of disease incidence as a discrete variable rather than the use of disease severity means that statistical procedures based on a normal distribution of the data cannot be applied. The spatial aggregation of diseased chicory heads within an experimental unit results in a tendency of the disease incidence towards relatively extreme disease incidence in an unbalanced frequency distribution. Hughes & Madden (1995a) have introduced the beta-binomial distribution to analyse aggregated data, to calculate indices of aggregation and to analyse the effect of treatments by model analysis. This study utilised their approach to investigate the effects of cultural practices on the disease incidence of bacterial softrot in witloof chicory.
As disease incidence data are discrete and binary, the binomial distribution can be used to describe the data. One underlying assumption of the binomial distribution is the constant probability for each individual to get diseased. The estimate of this probability is described by $p = X_i/n_i*N$ with X_i the number of diseased plants in sample i, n_i the number of plants per sample and N the total number of samples.

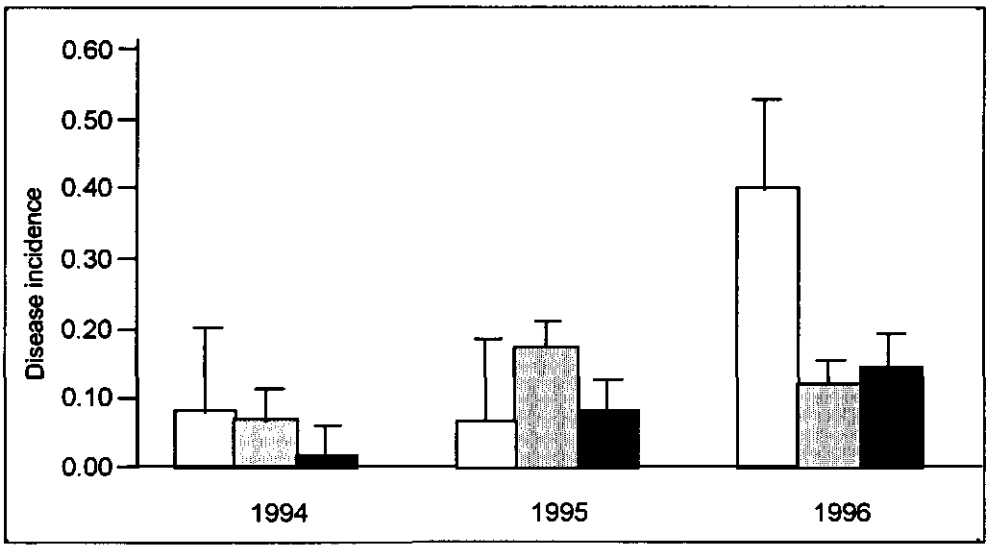

Fig. 1: Softrot incidence of chicory heads per tray at harvest. Data are averaged per forcing season (n = 24 in 1994 and 1996 and n = 20 in 1995). Forcing seasons are early (white), intermediate (light grey) and late (dark grey) forcing.

If the probability p of a plant being diseased is not constant, the choice of the binomial distribution is inappropriate. One of the reasons of a non-constant p is the tendency of individuals in a sampling unit to have similar disease status. This tendency leads to intracluster correlation or aggregation of disease. The probability p can be assumed to be a random variable with a beta probability density function and the distribution of the diseased plants can be described by the beta-binomial function with its additional parameter for aggregation, q.
Heterogeneity of the probability p increases as q increases, with relatively high probabilities near 0 and n (Hughes and Madden, 1995b). Increased probabilities near n and 0 imply that plants near a diseased plant have a greater probability to be diseased and plants near a healthy plant have a greater probability to stay healthy, the phenomenon called intracluster correlation or aggregation of disease.
For the analysis of variance of the disease incidence data of our experiments, the incidence data were logit-transformed. The statistical package Egret (1994) was used for the beta-binomial analysis.

Results

Disease incidence

Disease incidence increased for both temperature treatments during the three years, and incidence increased more in the high nitrogen, high temperature and low calcium treatments. Overall mean disease incidence ranged from values of 0.07 - 0.40 in the early forcing season to values of 0.02 - 0.17 in the intermediate and late forcing seasons (Figure 1).

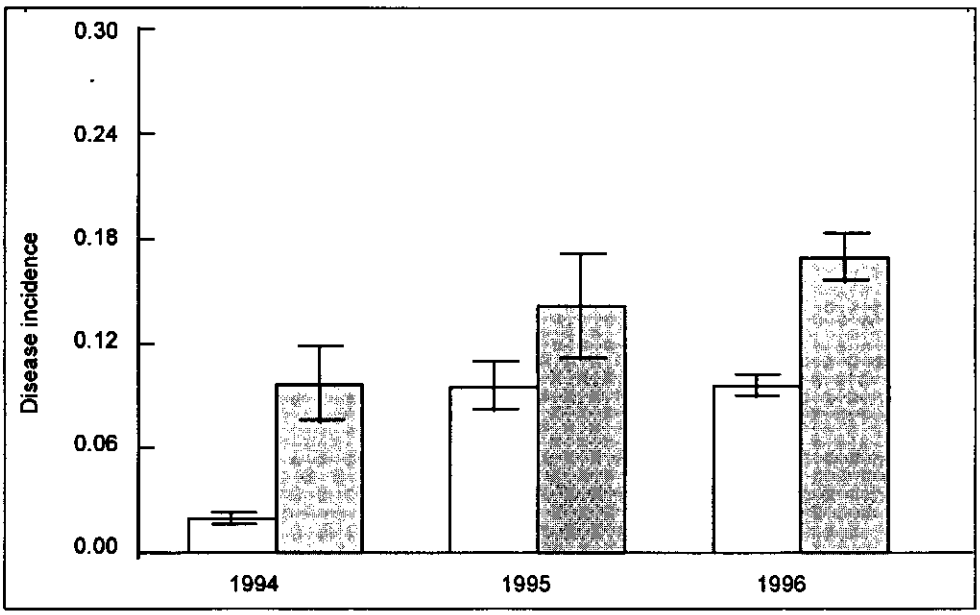

Fig. 2: Disease incidence of chicory heads per tray at harvest Data are averaged per forcing season at two different temperature regimes (n = 68), low (white) and high (grey) temperature.

The high temperature treatment resulted in higher disease incidences in all three years with the largest difference between temperature treatments in 1994 (Figure 2). Chicory heads from the low nitrogen treatment had lower disease incidences in both temperature treatments, ranging from 0.02 to 0.12. Disease incidence of chicory heads from the high nitrogen treatment were higher for both temperature treatments, ranging from 0.10 - 0.14.
The treatment combination of low temperature and high calcium resulted in the lowest disease incidence in all three years (0.04 in 1994, 0.12 in 1995 and 0.22 in 1996). Highest disease incidence was found in the treatment combination high temperature, low calcium and high nitrogen (0.23 in 1994, 0.26 in 1995 and 0.29 in 1996). At low forcing temperatures disease incidence was up to 50% lower than at high forcing temperatures. After calciumchloride treatment disease incidence was up to 60% lower than without. Variation of disease incidence of chicory heads within trays was higher in the high temperature (0.06-0.24), high nitrogen (0.00-0.18) and low calcium(0.09-0.18) treatments than in the low temperature (0.04-0.12), low nitrogen (0.00-0.11) and the high calcium(0.05-0.09) treatments.

Quality of the chicory heads

Correlations between disease incidence and the quality of the harvested crop were found for all treatments. Data of disease incidence and the fraction of chicory heads in quality class 1 were arcsin√-transformed to calculate Pearson's correlation coefficient, r.

Disease incidence and the fraction of chicory heads in quality class 1 were negatively correlated. Low temperature or low nitrogen treatments resulted in a lower disease incidence than high temperature and high nitrogen treatments. Only the disease incidence of chicory heads grown from roots of the high nitrogen treatment showed no significant correlation with quality. This indicates that quality may be high even in chicory heads form the high nitrogen treatment with a high disease incidence.

Analysis of variance – beta-binomial distribution

Frequency analysis of disease incidence showed a good fit of the observed data with the beta-binomial distribution (Figure 3). Goodness of fit for the beta-binomial distribution was highly significant with c2 = 17.8 (p = 0.003, n = 136) for high temperature data and c2 = 13.9 (p = 0.016, n = 136) for low temperature data.
Goodness of fit for the binomial distribution was not significant for either dataset (c2 =5.9 (p = 0.32) for high temperature data and c2 =8.3 (p = 0.14) for low temperature data).
The maximum-likelihood estimations for the treatment effects were significant for data of both temperature treatments (Table 1). Beta-binomial analysis for the datasets of the low and high temperature treatments showed the reducing effect of the high calcium treatment on disease incidence and the stimulatory effect of nitrogen on disease incidence.

Table 1: Parameter estimates and their standard errors (SE) for the beta-binomial model fitted to the logit of the disease incidence of chicory heads at low and high forcing temperatures (N=136). For each parameter, the statistical programme produces n-1 dummy variables, with n = 3 for year and season and n = 2 for calcium and Nitrogen. Estimates and standard errors of the dummy variables were calculated for the addition of a variable into the model.

Parameter	Low		High	
	Estimate	SE	Estimate	SE
Constant	-3.50	0.24	-1.90	0.21
Year				
1995	1.25	0.25	0.37	0.22
1996	1.99	0.24	1.49	0.20
Season				
2	n.s.[b]	n.s.	-0.50	0.20
3			-1.22	0.21
Calcium				
1	-0.57	0.17	-0.62	0.17
Nitrogen				
1	0.50	0.17	0.51	0.17
θ	0.11	0.02	0.15	0.02
Deviance [a]	433.40		481.80	

[a] Likelihood Ratio Statistic (LRS) of this model after inclusion of θ is significant at p < 0.001
[b] Inclusion of "season" is not significant (p>0.10) and therefore omitted.

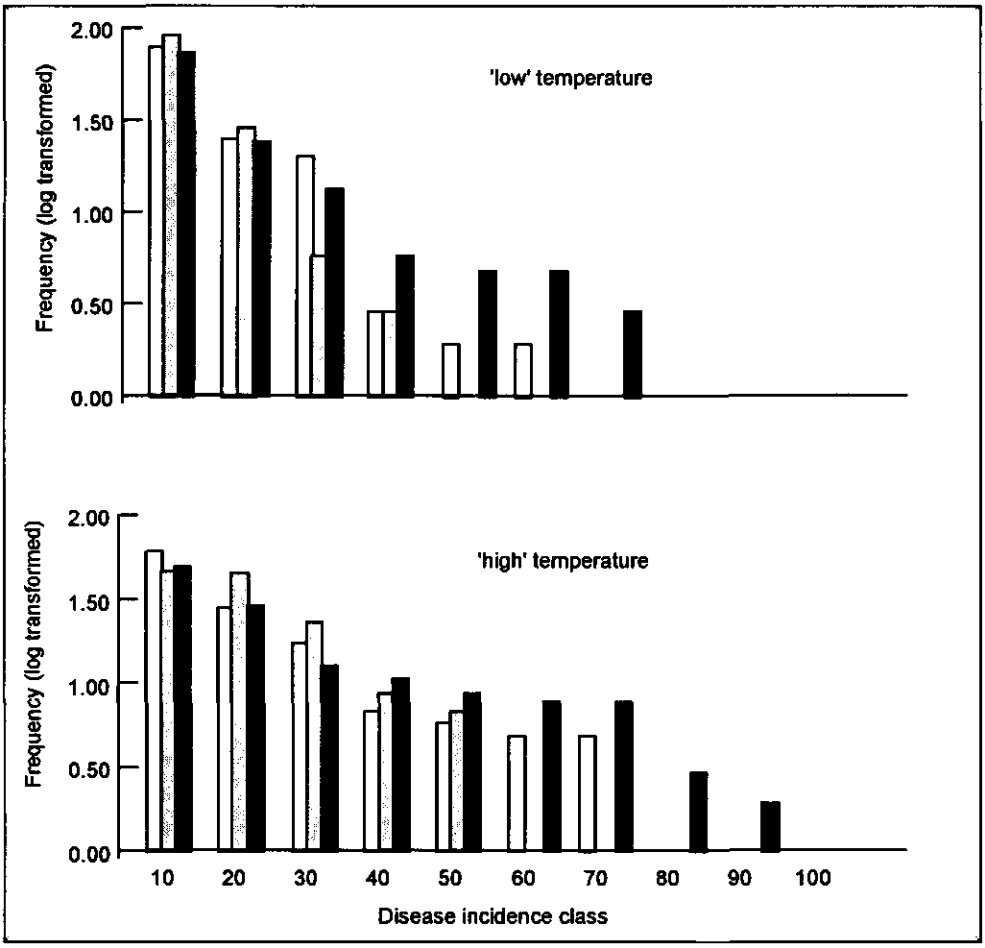

Fig. 3: Frequency distributions of observed and fitted disease incidence in 10 disease incidence classes for 'low' and 'high' forcing temperatures (n = 68). Frequencies are log-transformed for clarity. Class size is 10, with upper limit shown. White bars are observed frequencies, light grey bars expected frequencies for the beta-binomial distribution (θ = 0.15 for 'high' temperature and θ = 0.11 for 'low' temperature) and black bars expected frequencies for the binomial distribution (θ = 0, p = 0.12 for 'high' and p = 0.06 for 'low' temperature).

The model for the high temperature treatment showed a stronger effect of calcium and an additional effect of the forcing season. In both models, the aggregation parameter was highly significant ($p < 0.001$) indicating intracluster correlation of the disease. The aggregation parameter for disease incidence from low and high calcium treatments was higher for the high calcium($q = 0.32$) than for the low calcium($q = 0.26$) treatment.
The high calcium treatment not only resulted in a lower average disease incidence but also in an increase of the aggregation parameter q, which implies that the disease was more aggregated in trays with roots receiving high calcium treatment.

Discussion

Methodological comments

The opportunistic character of softrot bacteria in combination with the complicated production process of the crop resulted in a high aggregation level necessary for data analysis. The beta- binomial distribution could be applied to disease incidence data using forcing trays in the last production stage and not groups of individual chicory heads as the experimental and statistical unit to test for differences between treatments. Application of the beta-binomial distribution revealed the differences between the fertilisation and root immersion treatments applied in this experiments.
The production of witloof chicory can be divided into three distinct stages. The roots production in the field and the storage of the roots after defoliation never show symptoms of bacterial softrot although the bacteria can be readily detected on the crop leading to latent infection of the chicory plants. During the third stage of the production process, the forcing of the chicory heads, bacterial softrot will develop and will lead to symptoms of macerated plant tissue of the developing chicory heads. Chicory roots which are put to forcing are placed tightly into forcing trays which results in a dense packing of the growing chicory heads. The heads often touch each other which facilitates the spread of the bacteria and creates foci of disease, which results in clustering of the disease incidence, causes extra-binomial variation within the plots and over -dispersion of the data. The correction term used here to correct for the over-dispersion also allows the identification of the aggregation of the disease caused by experimental treatments.
The usefulness of applying the beta-binomial distribution could be demonstrated in this experiment. The model based on the beta-binomial distribution fitted the incidence data of both temperature treatments significantly better than the binomial distribution. The aggregation parameter θ in the beta-binomial model was shown to have a biological meaning in the aggregation of the disease within forcing trays. Treatments which reduced disease incidence, for example the immersion of the roots in calciumchloride, increased θ in the model.
The disadvantage of the model based on the beta-binomial distribution lies in the restriction of the statistical designs of experimental data which can be analysed with the available software. Model fitting using Egret is not possible with data from a split-plot design. The complicated production process of witloof chicory and the cultural practices studied here made such a model necessary.
As a consequence, the disease incidence data had to be split in two different datasets for the two temperature regimes under study. Nevertheless, the beta-binomial model was appropriate for both datasets and a comparison of the fitted models was possible. The biological interpretation of two coincident facts, reduction of disease incidence and increase of disease aggregation, is found in the phenomenon of focal spread of softrot. Conditions which reduce softrot lead to few and discrete foci. Conditions conducive to softrot lead to many, spreading and vaguely delimited foci, hence high incidence but low aggregation of the disease.

Pests and diseases can affect the crop during all three production stages of the crop and in our experiments measures were taken according to Dutch standard practice. Some of the observed diseases were Sclerotinia sclerotiorum and Phoma exigua. Apparently diseased roots were sorted out after harvest and remaining disease was treated. The application of vinchlozolin against Sclerotinia sclerotiorum has no known effect on bacterial softrot.

Pérombelon & Kelman (1980) defined two environmental conditions which affect the disease expression of softrot Erwinia's: the free water available on the plant surface and the minimum temperature required by the pathogen for its growth and infection of the plant. These conditions also had a significant effect on disease incidence in our experiments. The temperature of the processing water, the air temperature in the forcing cells and high relative humidity during forcing increased disease incidence.

Nitrogen fertilisation of the roots can result in a lower C/N ratio and subsequently in an increasing amino-acid content (Limami et al., 1993) and a reduced build-up of structural carbohydrates such as pectate (Reerink, 1993). Pectic substances are the initial substrate for the pectolytic enzymes of softrot bacteria (Pérombelon & Salmond, 1995) and the decrease in pectate partly explains the increased disease incidence in roots from the high nitrogen treatment.

Chicory heads produced in 1996 under high relative humidity showed a higher disease incidence at the three forcing periods (0.15-0.16) than heads produced under normal conditions (0.07-0.14). High relative humidity, in addition to the nutrient solution being warmer than the ambient air, resulted in a visible water film on the growing chicory heads. This water film will not only provide the bacteria with an entrance into the intercellular spaces but will also produce local patches with anaerobic conditions. In these patches, softrot bacteria of the genus Erwinia have an increased pathogenicity (Pérombelon, 1973) as they produce increased amounts of pectolytic enzymes. In addition, with a continuous water film and prevailing anaerobic conditions, only low amounts of bacteria (<102 cells) are needed to start tissue maceration (Pérombelon & Salmond, 1995).

Nitrogen fertilisation during root production contributed to favourable environmental conditions for bacterial infection by producing bigger and faster growing chicory heads, which became more densely packed in the forcing trays. Thus, the air circulation between the chicory heads was hampered and the relative humidity reached saturation. The metabolic activity of the growing plants increased the temperature above average air temperature. Together, these conditions increased the risk for bacterial softrot, which was translated in a higher disease incidence of chicory heads grown from roots produced under 'high' nitrogen levels and forced at 'high' temperatures.

The application of a 3% aqueous solution of calciumchloride, by inundation prior to forcing, was adapted from comparable measures in potato production (McGuire and Kelman, 1984). calcium reduced softrot incidence not only in potato (McGuire and Kelman, 1984, 1986), but also in apples (Conway, & Sams, 1987), other fruits (Conway, 1989) and beans (Bateman, 1964). calcium interacts with the polysaccharides in the cell wall, especially with the pectic substances. Incorporation of Calcium-ions into the pectic substances changes their three-dimensional structure. The pectinolytic enzymes of the bacteria cannot digest the substrate which its altered structure, so no breakdown of the plant cell walls will occur.

Subsequently, pathogenesis stops at the initiation of the disease. The effect is expressed as a reduced disease incidence after the calcium treatment in all three years of the experiment. In addition, the variation of the disease incidence within trays of the 'high' calcium treatment is smaller, suggesting a reduced spatial heterogeneity and spread of the disease.
The treatments applied in our experiments have been used in commercial forcing farms for several years. This study confirms the usefulness of selected cultural practices as a tool for disease control. In addition, the application of calciumchloride immersion of the roots does interact with the disease in a way, which can be quantified with the statistical methods applied here. The quantification of the effect of cultural practices can be utilised by the commercial chicory growers to reduce the risk of softrot when forcing roots with a known production history.

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Chapter 8

General discussion

General discussion

The results of the study on softrot in witloof chicory and its resulting implications are summarised and prospects for the management of the problem are discussed.

Aetiology and epidemiology

Softrot in witloof chicory has a disease cycle which is divided in two distinct stages of latent and symptomatic infection of the crop (Figure 1). During the root production, the crop is infected with softrot bacteria of the genera *Erwinia* and *Pseudomonas*. *Erwinia* stays latent in the crop, whereas *Pseudomonas marginalis* can cause a foliar disease, known as Marginal blight or Leaf necrosis. In the case of disease development, *Pseudomonas marginalis* will spread between chicory plants and form foci of disease (Schober & Zadoks, 1998). Latent populations of softrot bacteria spread less and the numerical increase of populations in the crop is mostly caused through migration from neighbouring crops and wild plants to chicory plants. Sources of decaying plant material, whether crops or wild plants, make up the major inoculum source for softrot bacteria. The harvest of the roots reshuffles the distribution of the softrot bacteria in the chicory crop as the plants are defoliated and the roots are trimmed. The wounds caused by these actions create entry points for the bacteria into the plant and softrot can develop. Redistribution may also be important for disease spread, either by the water film and leaking nutrient solution in the forcing trays or by wounds in the plant tissue caused by dense packing or anaerobic conditions. The cold storage of the roots after harvest will stop disease development and the softrot bacteria will fall back into a latent stage, unless the roots decay. During forcing of the roots for the chicory head production, softrot development will start again and accelerate on the growing chicory heads. Disease can spread rapidly in the warm and humid forcing cells, sometimes also transmitted by insect vectors. Infected chicory heads without visible symptoms of rot can develop softrot later in the post-harvest stage, during transportation, auction or shop display, when temperature changes cause condensation on the heads.

Chicory root production in the field

Witloof chicory plants are colonised by pectolytic bacteria during root production in the field. Although the bacteria do not cause disease, they establish populations which can cause disease later on in the production process. The number of pectolytic bacteria increases during the root production stage. Jaques *et al.* (1995) showed that the number of isolates of pectolytic bacteria does also increase on the leaf surface of broad-leaved endive (*Cichorium endivia* var. *latifolia*) throughout its growing season. He concluded that environmental conditions and the accessibility of leaves to migrating airborne or splashborne bacteria are major factors in the regulation of the population dynamics of pectolytic bacteria in the field. Most of the isolates of pectolytic bacteria can be found on the leaf base and near the stem in proximity to the soil (Tsuyama, 1974).

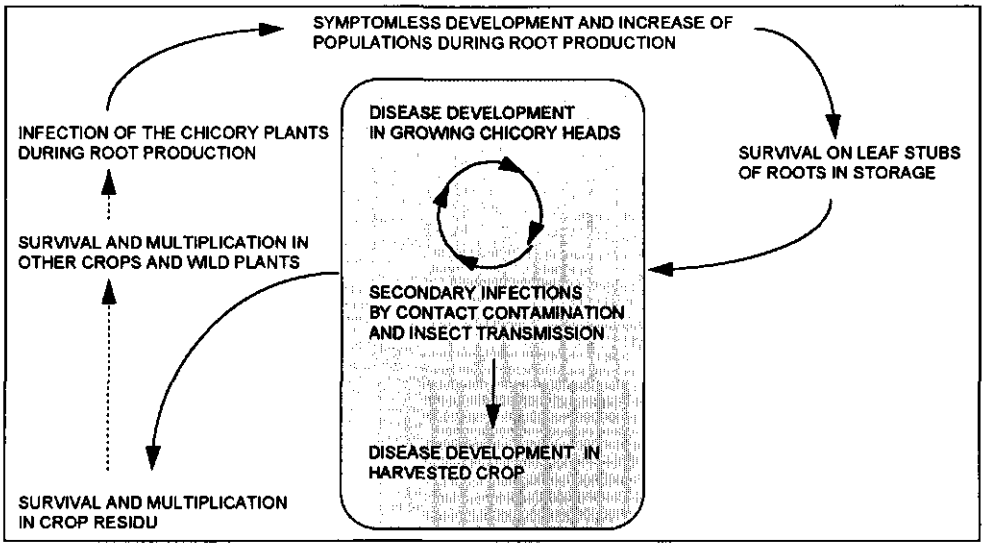

Fig. 1 Disease cycle of bacterial softrot in witloof chicory with latent (white) and symptomatic (grey) stages of the disease. Solid lines are established relations, dashed lines are putative.

A survey by De Boer (1983) showed rapidly increasing numbers of *E. carotovora* when susceptible tissue as potato vine debris became available. The bacteria switched from their saprophytic existence on the leaves to a pathogenic existence in the plant tissue. Witloof chicory leaves also ripen and decay at the end of the growing season, suggesting a similar switch in the behaviour of the bacteria. The suggestion is supported by the occasional occurrence of diseased plants in witloof chicory fields (but rarely in our field), caused by *Pm* and known as leaf necrosis or marginal blight.

The development of phytopathogenic species as saprophytes on the surface of witloof chicory leaves and root crowns has consequences for the later stages of the witloof chicory production, the storage and forcing of the roots. The number of softrot bacteria found on the leaves of the growing chicory plants was significantly and positively correlated with the average monthly temperature during the root production from May until late September. Environmental factors (rain, air humidity and radiation) showed no significant correlation with bacterial incidence in the present study. The ratio of isolates belonging to the genera *Pseudomonas / Erwinia* shifted towards 1 at harvest time in the favour of the *Erwinia's* (Chapter 5). As the canopy of the crop did not close before August, the sensitivity of the *Erwinia's* to high global radiation could be the factor inhibiting their growth in the early season. Ranganna *et al.* (1997) reported the total inhibition of softrot development in potatoes at UV-radiation levels of 15 kJ/m^2. After closure of the canopy, the water availability in the microclimate under and in the canopy differed from that at the top of the canopy.

The gradual change in the microclimate of the crop supposedly had a significant effect on bacterial numbers (Kinkel, 1997; Hirano *et al.*, 1995) and resulted in the observed increase of *Ecc*. Further analysis of the softrot severity during the root storage and forcing stages of the chicory production showed that the number of pectolytic isolates in the field affected the disease severity during forcing of the witloof chicory heads (Chapter 7).

Root storage

The colonisation incidence of stored witloof chicory roots by softrot bacteria attained its minimum in December, after 8-10 weeks of storage. Thereafter, the number of pectolytic isolates increased on the roots. Identification of the isolates showed that both Ecc and Pm contributed to these population changes (Chapter 6). The colonisation incidence by Ecc and Pm of chicory plants during storage by softrot bacteria increased each year due to a build-up of softrot bacteria at the field site. Pringle (1991) mentioned an increasing metabolic activity (sprouting) of the potatoes as a major factor for the increase in bacterial populations in storage. In witloof chicory, new metabolic activity of the stored roots is observed after December, expressed as growth of the apical bud and formation of new leaves (Chapter 6).

The crucial role of oxygen is emphasised by Pérombelon & Lowe (1975). Free water, caused by condensation on the surface of potato tubers, created local pockets of anaerobic conditions during storage where Ecc can multiply and cause disease. In addition, the anaerobic conditions can cause membrane instability in the tuber tissue leading to cell leakage. The leaked cell contents allow rapid growth of the bacteria. Water sprays applied during witloof chicory root storage supposedly create similar pockets of anaerobic conditions, enhancing the decomposition of the leaf stubs by facultatively anaerobic bacteria such as Erwinia (Goto, 1992).

The general decay of leaf stubs by several micro-organisms including fungi, emphasises another aspect of population change of softrot bacteria during storage. Saprophytic decomposition of the leaf stubs always involves fluorescent pseudomonads, the most common soil-borne bacterial species (Klement *et al.*, 1990). Strictly aerobic, they will persist during storage due to their strong saprophytic capabilities in aerobic spaces on the roots. The observed increase of the number of populations of Pseudomonas marginalis suggests that they can initiate softrot during forcing when present near wounded tissue of the growing witloof chicory head.

Forcing of chicory heads

Cultural practices not only influence the bacterial incidence during root production and storage, but have also a significant influence on disease severity after forcing. The amount of softrot after forcing of the roots was depended on forcing temperatures and the nitrogen application rate during root production (Chapter 7).
Low forcing temperatures and immersion of the roots in calciumchloride resulted in the lowest disease incidence. A reduction of up to 50% was found by using low forcing temperatures and a reduction up to 60% by application of calcium. In chicory heads grown from roots of the same field plots, disease incidence increased from the forcing period in October compared to the forcing period in December and March during our experiments (Chapter 7).

The observed decline during storage was not sufficient to reduce the numbers of bacteria carried along on the roots below the threshold levels needed for infection of the chicory heads during forcing. This evidence suggests that a population build-up in the field during root production can cause high disease incidence during forcing up to 5 months later, although the bacterial populations will decline in the intervening months of cold storage of the roots.

Where is the link – an attempt to explain softrot occurrence in witloof chicory

The presence of epiphytic populations of softrot bacteria does lead to disease when certain factors change in favour of the bacteria. These factors include environmental conditions, physiological stress of the host plant and the attainment of minimum threshold populations of softrot bacteria on or in the plant (Sigee, 1993).
In witloof chicory, these factors change when the roots are put to forcing. Water availability and ambient air temperatures reach levels which are suitable for bacterial multiplication. The relative humidity of the air is near saturation and allows the movement of the bacteria on the developing chicory heads. Due to the rapid growth of the developing chicory heads, small wounds will occur at the base of the heads. These wounds are invaded by the rapidly growing populations of softrot bacteria. Once the bacteria have gained access into the plant, they cause changes in the intercellular spaces.
The acidity of the environment will decrease causing cell membrane instability and cell leakage (Barras *et al.*, 1994). Locally occurring anaerobic pockets in and between the plants, caused by the relative humidity near saturation and increasingly denser packing of the growing chicory heads, will hamper plant defence mechanisms and enhance pathogenic activity of *Erwinia's* (Pérombelon & Salmond, 1994). Bacterial multiplication will increase further until their numbers reach threshold levels for the excretion of extracellular pectolytic enzymes (Barras *et al.*, 1994). Maceration of the plant tissue then is initiated and softrot will spread throughout any diseased chicory head. The metabolic activity of the bacterial populations increases the temperature and changes the acidity towards optimum levels for the bacteria, increasing bacterial spread and disease incidence.
The conditions applied during forcing of chicory cause physiological changes in the plant making it more susceptible for softrot and lessen plant defence mechanisms. For softrot bacteria the conditions change in favour of growth and spread, facilitating the establishment of new colonies and thereby increasing the probability of infection.
Bacteria not only spread through a water film on the plants but they are also transmitted by vector insects. Fruitflies of the genus *Drosophila* are common in chicory forcing cells (Boers, 1997) and our preliminary experiments have shown that these fruitflies may transmit softrot bacteria. The preferred feeding substrates of fruitflies are decaying and macerated plants. Deposition of softrot bacteria onto wounded tissue adds new infection sites increases disease incidence in a forcing cell and spreads softrot bacteria from one chicory head to the other.

Opportunities for disease Management

Disease management of bacterial disease is necessarily limited to sanitation and hygiene during the crop production process. In addition, the use of resistant cultivars and biological control measures may help to reduce disease.

Cultural practices

Nitrogen fertilisation during root production, high relative humidity during forcing and high temperatures of processing water and circulating air in the forcing cells increased disease incidence.

Nitrogen fertilisation of the roots can result in a lower C/N ratio and subsequently in a reduced build-up of structural carbohydrates such as pectate and cellulose (Reerink, 1993). Pectic substances are the initial substrate for the pectolytic enzymes of softrot bacteria (Pérombelon & Salmond, 1995) and the decrease in pectate partly explains the increased disease incidence in roots from the high nitrogen treatment. Pérombelon and Kelman (1980) defined the environmental conditions affecting the disease expression of softrot *Erwinia's* as free water available on the plant surface and temperatures above the minimum required for growth and infection. These conditions also had a significant effect on disease incidence and severity in our experiments.

Chicory heads produced in our experiments in 1996 under high relative humidity showed a higher disease incidence at the three forcing periods than heads produced under low humidity. High relative humidity, along with the nutrient solution being warmer than the ambient air, resulted in a visible water film on the growing chicory heads. The water film not only provided the bacteria with an entrance into the intercellular spaces but also produced local patches with anaerobic conditions. In these patches, softrot bacteria of the genus *Erwinia* have an increased pathogenicity (Pérombelon, 1973) as they produce increased amounts of pectolytic enzymes. In addition, with a continuous water film and prevailing anaerobic conditions, only low amounts of bacteria ($<10^2$ cells) are needed to initiate tissue maceration (Pérombelon & Salmond, 1995). Nitrogen fertilisation during root production contributed to environmental conditions favourable for the bacterial infection process by producing bigger and faster growing chicory heads, which became more densely packed in the forcing trays than the omission of nitrogen fertilisation.

Thus, the air circulation between the chicory heads was hampered and the relative humidity reached saturation. The metabolic activity of the growing plants increased the temperature above average air temperature. Together, these conditions increased the probability of softrot development, which was translated into a higher disease incidence of chicory heads grown from roots produced under 'high' nitrogen levels and forced at 'high' temperatures. The effect is expressed as a reduced disease incidence after the calcium treatment in all three years of the experiment. In addition, the variation of the disease incidence within trays of the 'high' calcium treatment is smaller, suggesting a reduced spatial heterogeneity and less spread. Subsequently, pathogenesis stops at the initiation of the disease.

The treatments applied in our experiments have been used in commercial forcing farms for several years. Our study confirms the usefulness of cultural practices as a tool for disease control. In addition, the application of calcium chloride immersion of the roots does interact with the disease in a way which can be quantified with the statistical methods applied here. Results of the quantitative studies on the effect of cultural practices can be used by the commercial chicory growers to reduce the risk of softrot when forcing roots with a known production history.

The application of a 3% aqueous solution of calcium chloride by inundation before forcing was adapted from comparable measures in potato production (McGuire and Kelman, 1984). Calcium interacts with the polysaccharides in the cell wall, especially with the pectic substances. Incorporation of Calcium ions into the pectic substances changes their three-dimensional structure. The pectolytic enzymes of the bacteria cannot digest the substrate which its altered structure, so that no breakdown of the plant cell walls will occur.

Resistance

Chicory cultivars have an inherent resistance to softrot. The usage of two cultivars, one susceptible and one more resistant under the same forcing conditions, may result in 10 per cent more softrot in the susceptible cultivar (Chapter 3).

The cultivar specific nitrogen uptake and the correlation between nitrogen content of the chicory roots and susceptibility for softrot may explain the varying resistance between chicory cultivars (Reerink, 1993). Wolters and Collins (1995) showed that resistance to softrot in potato is correlated to calcium uptake, suggesting a direct link between pectate composition (the primary substrate for softrot bacteria) and susceptibility.

Chicory cultivars vary in their susceptibility to softrot. The variation is caused by two very distinct characteristics of the cultivars. First, cultivars are bred for one of the three specific forcing seasons, that is early after harvest, intermediate between November and March and late after March until September. In general, the susceptibility to bacterial softrot decreases with the proceeding forcing season (Van Kruistum, 1997). Most softrot is found early in the season (Chapter 7). Cultivars used for intermediate and late forcing will develop less softrot than cultivars used for early forcing. Attempts to combine softrot resistance with the possibility to use the cultivar for early forcing have proven futile until today.

Plant elicitors play a crucial role in induced disease resistance. Metabolites of pectic degradation of the plant cells by bacterial enzymes may act as elicitors for disease resistance (Vidal *et al.*, 1998)). Significant reduction of the proportion of diseased potato tissue was observed when the tubers where treated with oligo-galacturonides before artificial infection with *Ecc* (Dutton *et al.*, 1997). As disease development is favoured under conditions adverse to the expression of disease resistance (Pérombelon and Salmond, 1995), it is unlikely that naturally occurring levels of elicitors or plant defence mechanisms are adequate to reduce the disease efficiently. Currently the most realistic disease controls might be a combination of cultural practices and monitoring the colonisation incidence during root storage. Cultural practices known to reduce disease risk in combination with periodical screening of the crop might lead to a tool for chicory growers. It would help to assess the risk for bacterial softrot for each separate lot of chicory roots intended for forcing. As a consequence, all parties involved in the production chain should participate in a system for quality control of their respective product, harvested roots, stored roots or chicory heads.

Biological control

Some attempts have been made to identify antagonists of softrot bacteria. Low bacterial densities of epiphytic populations may be controlled by naturally occurring saprophytic micro-organisms on the plant surface. Experiments on sweet pepper, *Capsicum annuum* L. (Melo, 1995) identified several fluorescent pseudomonads and yeasts as antagonists of *Erwinia carotovora*.

Softrot in sweet pepper could be reduced by applying naturally occurring yeasts of the genus *Rhodotorula* or the bacterium *Pseudomonas fluorescens* isolate LD-4. These micro-organisms were able to decrease the lesion size by 95-98% though they did not influence the growth of *Erwinia carotovora in vitro*. Melo concluded that the most probable mode of action of the antagonists is competition.

Application of these antagonists in combination with calcium reduced the effect of the antagonists, which makes a combination of disease control measures in sweet pepper hazardous.

Yeasts and fluorescent pseudomonads naturally occur on chicory (Van Outryve *et al.*, 1989) and may contribute to control latent populations of softrot bacteria on the plant surface. Little is known, however, on their population dynamics and growth requirements. Van Outryve found populations of saprophytic pseudomonads on the leaves of normally forced chicory heads which suggest that these antagonists are capable of surviving the root storage period. Research in this area is highly recommended as it could give chicory growers an opportunity to control bacterial softrot before it is manifest. Roots are already treated against several fungal diseases and Calcium immersion is used widely. Adding antagonists as a biological control agent could be an integrated treatment of the roots without the necessity of additional equipment or time intensive applications.

Conclusions

Bacterial softrot is a common microbiological process in nature, being part of the turn-over of organic matter. The decomposition of biomass is an integral contribution to the recirculation of nutrients and minerals. The decay of senescent plant parts is the major contributor to this decomposition process. Wild plants delay microbial brake down with plant-defence mechanisms.

The internal plant structure is a mechanical barrier against microbial attack. Intensive production processes, such as the forcing of chicory, do not allow the time necessary for the plant to build these mechanical barriers. The balance between plant growth and microbial attack will tip in favour of the micro-organisms and disease will develop. Chicory heads showed little or no bacterial softrot before the introduction of the hydroponics system for forcing (Vantomme *et al.*, 1989). Chicory farmers applying a the intensive forcing method using the hydroponics system have one option to control disease. They can eliminate the cause of the disease, the vulnerability of the growing chicory heads due to the physiological and environmental condition of the plant making it vulnerable to bacterial attacks. By strengthening the mechanical barriers of the plants and by creating conditions unfavourable for bacterial spread, plant defence mechanisms will control microbial growth and disease development. Farmers must learn to balance between the maximum attainable yield in the shortest possible time and crop production maximising plant health.

Recommendations for research

Fundamental research	Applied research
Biological control of softrot bacteria by antibiotic producing pseudomonads and plant elicitors.	Quantification of the effect of fruitflies on bacterial softrot incidence during forcing.
Mechanisms of resistance in chicory cultivars and wild *relatives (Cichorium intybus* L. var. *foliosum* DC).	Optimising the effect of calciumchloride on reducing bacterial softrot during forcing.
Modelling population dynamics of the softrot bacteria in the field and during storage in relation with key environmental factors.	Scenario studies on the effect of latent populations of softrot bacteria ion the field on disease incidence during forcing.
Interactions of softrot bacteria with other pests and diseases and the impact on plant health.	

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Summary

Chapter 1 - General introduction

Softrot of witloof chicory is caused by several phytopathogenic bacteria, of which Erwinia carotovora subsp. carotovora and Pseudomonas marginalis are found in the Netherlands. The initial colonisation of the chicory plants happens before the harvest of the roots, during the field season of the root production stage. The pathogens are introduced into the forcing cells on the roots and leaf stubs and spread from one chicory head to another, generally causing foci of disease in the forcing trays. To prevent the disease, several measures can be taken including cultural practices during the root production, the storage and the forcing of the crop, hygiene at harvest and adjusted handling techniques of the crop. Bacterial softrot is caused by bacteria with a wide host range and an ability to adapt to a wide range of environmental conditions so that the design of new measures for the reduction of disease incidence and severity is complicated. We investigated the importance of cultural and environmental factors on disease incidence and severity. Several aspects from the three production stages of the chicory production were chosen to be studied in detail. The influence of nitrogen management and environmental factors such as temperature and radiation during root production were studied for their effect on the occurrence of softrot bacteria in the field as latent infections. Temperature and relative humidity during root storage were chosen as they determine the probability for survival of the softrot bacteria on the crop. The application of calciumchloride to the roots after harvest and the forcing conditions among which temperature and humidity are known to affect bacterial softrot in witloof chicory during forcing. The regulation of the softrot incidence and severity during forcing and the influence of calciumchloride and environmental conditions as well as cultural practices were investigated.

Chapter 2 - Detection of softrot bacteria

We developed a technique combining spiralplating and immunofluorescence colony staining (IFC) which offers maximum recovery and qualitative assessment of softrot *Erwinia's*.

This technique was evaluated for the soft-rot pathogen *Erwinia carotovora* subsp. *atroseptica* in witloof chicory. Target bacteria can be detected, quantified and their numbers can be related to the total number of micro-organisms present in a sample. Target bacteria could be detected in platings at various dilutions of plant washings. The combination of spiralplating and IFC proved to be a functional tool for quantification of target and non-target bacteria and the isolation of target bacteria as pure cultures from IFC-positive colonies. It reduced the amount of conjugate necessary for staining target bacteria compared to the traditional IFC technique and results showed little variation within replications. Recovery of target bacteria both in pure cultures and in plant washings, was significantly higher than with traditional media.

Chapter 3 - Enzymatic maceration of witloof chicory

The progress of bacterial softrot in witloof chicory heads and the activity of pectolytic enzymes of the softrot pathogen *Erwinia carotovora* subsp. *carotovora* were studied in several witloof chicory cultivars. Disease incidence, the proportion of diseased chicory heads in a forcing tray after forcing of the heads, was assessed during three forcing seasons per year. Nitrogen, calcium and the forcing season had a significant effect on disease incidence in witloof chicory cultivar Rumba. Activity of the pectolytic enzymes polygalacturonase (PG) and pectate lyase (PL) in artificially inoculated chicory heads of cultivar Rumba reached their maximum after each other, PG showed highest activities 48 hours after inoculation and PL 72 hours after inoculation. Maceration of the chicory tissue and bacterial growth increased continuously until 96 hours after inoculation, when more than 60% of the chicory heads was macerated by pectolytic enzymes of the bacteria.

Enzyme activity of *Ecc* in cell wall extracts from chicory cultivar Rumba was not influenced by the nitrogen and calcium treatment of the chicory plants. PL activity decreased continuously until 72 hours after inoculation. Activity of PG reached its highest levels 48 hours after incubation. Calcium affected the enzyme activity of both pectolytic enzymes in the low nitrogen treatment 24 hours after inoculation. Bacterial growth was not affected by the calcium or nitrogen treatment.

Significant differences in susceptibility to bacterial softrot were found for the tested chicory cultivars. Disease severity after 96 hours ranged from 6% in Clause R2 to 100% in Bea and Tabor. Chicory cultivars Rumba and Salsa showed an average severity of 65 - 70%. Bacterial softrot progressed most rapidly in cultivar Salsa and progressed slowest in cultivar Clause R2.

Chapter 4 – Influence of temperature and water availability

The effect of the water availability and the temperature of the growth substrate on growth and disease development of softrot bacteria were studied using artificial media and plant material. Water availability was measured as the osmotic potential of a solution (ψ_{osm}) and was assessed for solutions of PEG4000 and KNO_3 as artificial osmotica and for plant tissue of chicory heads. Growth of the softrot bacteria *Erwinia carotovora* subsp. *carotovora*, *Erwinia carotovora* subsp. *atroseptica* and *Pseudomonas marginalis* was found at water potentials from -0.12 MPa to -8.00 MPa. The lag phase of the growth curve increased with decreasing water potential. The relative growth rates of the three softrot pathogens showed a sigmoidal relationship with water potential, the relative growth rates decreasing rapidly at water potentials lower than -1.5 MPa. The water potential of harvested chicory heads decreased with storage time of the harvested crop but was still within the growth limits for softrot bacteria. In relation to temperature, the relative growth rate of *Ecc* was highest at 10°C, of *Eca* at 15°C and of *Pm* at 5°C. Chicory heads of two chicory cultivars, Rumba and Salsa, inoculated with *Ecc* had a significantly higher severity at 30°C (0.72 for Rumba and 0.47 for Salsa) than at lower or higher temperatures. In conclusion, temperature and water availability found during forcing of chicory are not limiting factors for maintaining populations of softrot bacteria. Possibilities for crop protection thus are only available during chicory root storage. During the storage a low growth rate of the softrot bacteria may result in a decrease of bacterial populations below the minimum densities needed for infection during forcing of the chicory heads.

Chapter 5 - Softrot bacteria during chicory root production in the field

Witloof chicory plants were surveyed during the root production in the field for the presence softrot bacteria on the chicory leaves in a 4-year field experiment.

The bacteria can be present as epiphytic or saprophytic populations and consist, in the Netherlands, of the species *Erwinia carotovora* and *Pseudomonas marginalis*. Isolates of pectolytic bacteria collected from the leaves were identified and the influence of environmental variables was assessed testing the hypothesis of an increase of the number of isolates on the chicory leaves. A significant linear correlation was found for the number of pectolytic isolates and time in months and temperature (monthly average). The average monthly growth rate (r) of the softrot bacteria was significantly greater than zero during the field season, indicating an increase of the populations of softrot bacteria. The ratio between the number of *Erwinia* and *Pseudomonas* isolates changed during the growing season from a predominance of pseudomonads from May until July to an equal ratio from August until harvest. The number of isolates of *Erwinia carotovora* was negatively correlated with total global radiation. The increase in the number of *Erwinia carotovora* later in the growing season was attributed to protection from high radiation levels and adverse temperatures in the microclimate under the canopy.

Chapter 6 - Softrot bacteria during storage of chicory roots

Changes in the populations of softrot bacteria occurred during the cold storage of witloof chicory roots. Populations of *Erwinia carotovora* subsp. *carotovora*, *Erwinia carotovora* subsp. *atroseptica* and *Pseudomonas marginalis* were followed from October (directly after harvest) until March of the next year. The colonisation incidence of *Erwinia carotovora* subsp. *carotovora* and *Pseudomonas marginalis* decreased during the early weeks of storage and then significantly increased after December until the end of the storage period in March. This pattern occurred in each of the three sampling years. During the storage period in 1996, populations of *Eca* decreased continuously until mid-November without any further changes during the rest of the storage period. Time-course analysis of the monthly colonisation incidence of *Ecc* and *Pm* over the three years revealed a significant dependency of the incidence on the incidence two weeks earlier. In addition, high colonisation incidences during storage coincided with high disease incidence during the following forcing period of the roots.

Therefore, a forecast of disease incidence for the forcing of the witloof chicory heads based on the colonisation of the roots during the storage period may be envisaged.

Chapter 7 - Softrot bacteria during forcing of the chicory heads

Witloof chicory can develop a slimy softrot during the last days of the forcing period, which may induce losses up to 60% of the crop. The influence of cultural practices on disease incidence and the aggregation of the disease were studied in a three year experiment at two different temperatures. The mean disease incidence was lowest in 1994 and highest in 1996 and ranged from 0.07 - 0.13 in the early forcing season to 0.15 - 0.18 in the two later seasons. Chicory heads forced at low temperatures and grown from roots receiving low nitrogen fertilisation or immersed in calciumchloride had a relatively low disease incidence. Disease incidence was aggregated and followed a beta-binomial distribution. Chi-square analysis for goodness of fit for the beta-binomial distribution was highly significant with $\theta = 0.15$ ($n = 136$, $p = 0.003$) for the high temperature and with $\theta = 0.11$ ($n = 136$, $p = 0.016$) for the low temperature treatment during forcing. Calcium chloride application as a root immersion treatment and low nitrogen levels during root production decreased the disease incidence and increased the aggregation parameter θ. The disease formed smaller foci in chicory heads from these treatments during forcing, which resulted in a reduced spread of the disease within the forcing trays.

8. General discussion

The presence of epiphytic populations of softrot bacteria on witloof chicory plants can lead to disease when certain factors change in favour of the bacteria. Factors include environmental conditions, physiological stress of the host plant and the attainment of minimum threshold values for populations of softrot bacteria on or in the plant. Factors change when the roots are put to forcing. The ambient air temperatures and the relative humidity reach levels favourable for the creation of a water film on the crop suitable for bacterial multiplication. Softrot bacteria not only spread through this water film on the plants but are also transported by vector insects. Deposition of softrot bacteria onto wounded tissue by fruitflies does create new infection sites, spreading softrot bacteria from one chicory head to the other.

Chicory farmers then have one option to control the disease by altering the environmental conditions which favour bacterial multiplication and make the chicory plants vulnerable for a bacterial attack. Strengthening the mechanical barriers of the plants using low nitrogen doses for fertilisation and by preventing bacterial spread in the forcing cells plant defence mechanisms can be relied upon to control microbial growth and disease development.

Zusammenfassung

Zichoriepflanzen wurden während der gesamten Produktion von Zichorien auf den Befall mit Naßfäulebakterien untersucht. Bei dem Produktions-prozeß des Zichorie unterscheidet man drei Phasen, die Wurzel-produktion, die Lagerung der Wurzeln und die eigentliche Treiberei der Zichoriesprossen. In den Niederlanden wird die bakterielle Naßfäule verursacht durch die Erreger *Erwinia carotovora subsp. carotovora* und *Pseudomonas marginalis* verursacht. Populationen dieser Erreger kolonisierten die Blätter der Zichoriepflanzen während der Wurzel-produktion im Freiland. Naßfäule entstand hingegen nur während der Treiberei. Die Zahl der Populationen der Naßfäulebakterien auf den Zichorieblättern, nahm während der Wurzelproduktion zu, obwohl *E. carotovora* durch hohe Strahlungsintensitäten während der ersten Monate der Wurzelproduktion in seinem Wachstum eingeschränkt wurde. Die Zahl der Naßfäulebakterien wurde durch die Temperatur in der Wachstumsperiode der Zichoriewurzeln, aber nicht durch den Niederschlag oder die Stickstoffdüngung beeinflußt. Während der Lagerung der Zichoriewurzeln nahm die Zahl der Naßfäulebakterien vom September, direkt nach der Ernte, bis zum Dezember kontinuierlich ab um danach wieder zu zu nehmen bis zum Ende der Lagerungsperiode im März. Die relative Luftfeuchte in der Lagerräumen hatte einen signifikanten Einfluß auf das Vorkommen der Naßfäulebakterien und ein erhöhtes Vorkommen der Bakterien während der Lagerungsperiode war mit einem vermehrten Auftreten der Naßfäule während der nächstvolgenden Treiberei korreliert. Bakterielle Naßfäule wurde in den drei untersuchten Treibperioden wahrgenommen. Der stärkste Befall wurde während der frühen Treibperiode im Oktober/November und der Treibperiode im Dezember/Januar wahrgenommen. Während der späten Treibperiode im März trat die Krankheit nicht so stark auf. Die Befallsintensität war von der Stickstoffdüngung der Pflanzen im Freiland, der Calciumbehandlung der Wurzeln und dem Versuchsjahr abhängig. Die Naßfäule trat in den Zichoriepflanzen in den Treibkisten in aggregierten Krankheitsherden auf. Die Calciumbehandlung der Wurzeln vor der Treiberei verursachte eine verstärkte Aggregation der Krankheitsherde, wodurch sich die Krankheit nicht so stark ausbreiten konnte wie in den Treibkisten mit unbehandelten Pflanzen. Hohe Stickstoffdüngung der Wurzeln förderte das Auftreten der Krankheit während der Treiberei und verursachte größere Krankheitsherde in den Treibkisten. Sowohl die Wirkung des Stickstoffs als auch des Calciums konnte durch die Aktivität pektolytischer Enzyme der Naßfäulebakterien erklärt werden. Diese Enzyme greifen die Zellwände der Zichoriepflanzen an. Calcium wird in den Zellwänden gebunden wodurch die Aktivität der bakteriellen Enzyme abnimmt. Bakterielle Naßfäule konnte durch einen geringere Stickstoffdüngung während der Wurzelproduktion und durch eine Tauchbehandlung der frisch geernteten Wurzeln in einer Calciumchlorid-Lösung reduziert werden. Beide Maßnahmen stärkten auch den Widerstand heranwachsende Zichoriesprossen gegen die Enzyme der Bakterien und damit wurde das Wachstum der Bakterien reduziert und die Entwicklung der Krankheit gehemmt.

Samenvatting

Hoofdstuk 1 - Algemene inleiding

Natrot in witlof wordt door verscheidene plantpathogene bacteriën veroorzaakt. *Erwinia carotovora* subsp. *carotovora* en *Pseudomonas marginalis* zijn in Nederland het meest belangrijk. Het gewas wordt tijdens de penproduktie voor de oogst gekoloniseerd door de bacteriën. De pathogenen komen op de pennen zelf en de bladkraag in de witloftrek terecht en veroorzaken ziektehaarden in de trekbakken, waarbij zich de ziekte tussen de witlofkroppen verspreidt. Om de ziekte te beheersen, kunnen teeltmaatregelen tijdens de wortelproduktie, de bewaring van de pennen en tijdens de trek zelf aangepast worden. Hygiënische omgang met het gewas tijdens de oogst en in de na-oogstfase zelf zijn belangrijk om verdere besmetting van het gewas te voorkomen. De brede waardplanten-reeks van de natrotbacteriën en hun aanpassingsvermogen aan hun omgeving maken het moeilijk om nieuwe bestrijdingsstrategieën tegen de ziekte te ontwikkelen met een verminderde kans op besmetting en een geringere ziektegraad. Aspecten van de drie produktiestappen van de witlofproduktie werden nader onderzocht. Zo werden de invloeden van de stikstofbemesting en de omgevingsfactoren temperatuur en straling tijdens de penproduktie op het voorkomen van latente populaties van natrotbacteriën op het gewas onderzocht. De bewaartemperatuur van de pennen en de luchtvochtigheid tijdens de bewaring beïnvloeden de overlevingskansen van de bacteriën tijdens de bewaring. Deze factoren werden daarom gekozen voor nader onderzoek, alsook de invloed van een calciumbehandeling van de pennen en de omstandigheden tijdens de trek. Van de laatste twee factoren is bekend dat ze de mate van aantasting kunnen beïnvloeden, en daarom werd hun uitwerking op het voorkomen van de ziekte bestudeerd.

Hoofdstuk 2 - Aantonen van natrotbacteriën

Het doel van deze studie was de ontwikkeling van een techniek waarmee natrotbacteriën aangetoond kunnen worden. De combinatie van "spiralplating" met immunofluorescentie-kleuring bood de mogelijkheid om de hoogst haalbare recovery te combineren met de kwalitatieve beoordeling van natrotbacteriën van het genus *Erwinia*. De techniek werd voor de natrotbacteriën *Erwinia carotovora* subsp. *atroseptica*, het meest voorkomende natrotpathogeen in België en Frankrijk, in witlof geëvalueerd. Doelbacteriën konden aangetoond, gekwantificeerd en aan het totale aantal micro-organismen van een monster gerelateerd worden. De natrotbacteriën werden in suspensies afkomstig van witlofpennen en kroppen aangetoond. De ontwikkelde techniek bleek een werkbare methode om zowel natrotbacteriën als ook andere micro-organismen in en op witlofplanten te kwantificeren. De natrotbacteriën konden met deze techniek in reincultuur gebracht worden. De techniek verbruikte minder geconjugeerd antiserum dan de traditionele immunofluorescentie-kleuring en de resultaten vertoonden doorgaans een zeer geringe spreiding tussen de herhalingen. De recovery van natrotbacteriën zowel uit reinculturen als vanuit planten was significant hoger dan met gangbare selectieve media gerealiseerd kon worden.

Hoofdstuk 3 – Enzymatische maceratie van witlof

De ziekte-incidentie van bacterieel natrot in witlof en de activiteit van pectolytische enzymen van *Erwinia carotovora* subsp. *carotovora* werden onderzocht. De ziekte-incidentie na de trek was afhankelijk van de stikstof- en de calciumbehandeling van de witlofplanten, uitgevoerd voor de trek. Stikstof, calcium en het trekseizoen beïnvloedden de ziekte-incidentie significant bij de witlofcultivar Rumba. De pectolytische enzymen polygalacturonase (PG) en pectaatlyase (PL) in geïnoculeerde witlofkroppen van de cultivar Rumba bereikten na elkaar hun maximale activiteit, PG na 48 uur en PL na 72 uur. De maceratie van het witlofweefsel en de groei van de bacteriën namen tot 96 uur na inoculatie continu toe. Meer dan 60% van de witlofkroppen was 96 uur na inoculatie gemacereerd. De enzym-activiteit van *Ecc* in celwandextracten van de witlofcultivar Rumba werd niet door de stikstof- en calciumbehandelingen van de witlofkroppen beïnvloed. De

activiteit van PL nam na inoculatie continu af tot 72 uur na inoculatie en de activiteit van PG bereikte na 48 uur haar maximum. 24 uur na inoculatie beïnvloedde calcium bij de lage stikstof-behandeling de activiteit van beide enzymen significant. De gevoeligheid voor natrot was voor de onderzochte witlofcultivars significant verschillend. De ziektegraad 96 uur na inoculatie varieerde van 6% in cultivar Clause R2 tot 100% in Bea en Tabor. De witlofcultivars Rumba en Salsa waren gemiddeld 65-70% aangetast.

Hoofdstuk 4 – Invloed van temperatuur en waterbeschikbaarheid

De invloeden van waterpotentiaal en temperatuur van voedingsbronnen op de groei en de ziekteontwikkeling van natrotbacteriën werden bestudeerd met behulp van kunstmatige media en plantenmateriaal. De waterbeschikbaarheid werd als het osmotisch potentiaal van een oplossing (ψ_{osm}) gemeten. Hiervoor werden oplossingen van PEG4000 en KNO_3 en plantemateriaal als voedingsbronnen gebruikt. Groei van de natrotbacteriën *Erwinia carotovora* subsp. *carotovora (Ecc)*, *Erwinia carotovora* subsp. *atroseptica (Eca)* en *Pseudomonas marginalis (Pm)* werd gevonden bij waterpotentialen tussen –0.12 MPa en –8.00 MPa, maar de lag-fase van de groeicurve nam toe met afnemend waterpotentiaal. De relatieve groeisnelheden van de drie natrotbacteriën vertoonden een sigmoidale relatie met de waterpotentiaal en daalden bij waterpotentialen lager dan –1.5 MPa. De waterpotentiaal van geoogste witlofkroppen nam tijdens de bewaarperiode toe maar bleef binnen de grenzen nodig voor de groei van de bacteriën. De relatieve groeisnelheden van de bacteriën bij verschillende kweektemperaturen waren voor *Ecc* bij 10°C, voor *Eca* bij 15°C en voor *Pm* bij 5°C het hoogst. Witlofkroppen van de cultivars Rumba en Salsa, geïnoculeerd met *Ecc*, vertoonden de hoogste ziektegraad bij 30°C (0.72 voor Rumba en 0.47 voor Salsa). De temperaturen en waterpotentialen zoals tijdens de trek van witlof gebruikelijk beperkten de groei van natrotbacteriën niet. Mogelijkheden voor gewasbeschermings-maatregelen waren er daarom alleen tijdens de bewaring van de witlofpennen. Tijdens de bewaarperiode kon een lage groeisnelheid van de natrotbacteriën tot een afname van de bacteriepopulaties tot onder het minimum aantal bacteriën leiden dat voor een geslaagde infectie nodig is.

Hoofdstuk 5 - Natrotbacteriën tijdens de wortelproduktie op het veld

Witlofplanten werden tijdens de wortelproduktie in een vierjarig veldexperiment onderzocht op het voorkomen van epifytische populaties van natrotbacteriën. Natrotbacteriën konden als epifytische of saprofytische populaties aanwezig zijn. De populaties bestonden in Nederland uit de soorten *Erwinia carotovora* en *Pseudomonas marginalis*. Isolaten afkomstig van witlofblad werden geïdentificeerd en de invloed van omgevingsfactoren op hun aantallen werd onderzocht. Hierbij werd uitgegaan van de hypothese dat het aantal isolaten tijdens het groeiseizoen op het witlofblad toeneemt. Het aantal isolaten was lineair en significant gecorreleerd met de maand van het groeiseizoen en de gemiddelde temperatuur per maand. De gemiddelde groeisnelheid van de natrotbacteriën was tijdens het groeiseizoen significant hoger dan nul. Dit wijst erop dat de populaties in grootte en aantal toenamen. De verhouding tussen de aantallen *Erwinia* en *Pseudomonas* isolaten veranderde van overwegend *Pseudomonas* van Mei tot Juli tot een gelijke verhouding tussen beide genera van Augustus tot aan de oogst. Het aantal *Erwinia* isolaten was negatief gecorreleerd met de globale straling. De toename van *Erwinia carotovora* in het latere groeiseizoen werd aan de beschermende werking van het microklimaat onder het gewas toegeschreven. Dit microklimaat beschermde de bacteriën voor hoge stralingsintensiteiten en ongunstige temperaturen.

Hoofdstuk 6 - Natrotbacteriën tijdens de bewaring van de pennen

De populaties van natrotbacteriën veranderden tijdens de bewaring van witlofpennen. Populaties van *Erwinia carotovora* subsp. *carotovora*, *Erwinia carotovora* subsp. *atroseptica* en *Pseudomonas marginalis* werden vanaf Oktober, direct na de oogst, tot Maart van het volgende jaar gevolgd. Pennen werden in 1996 met *Erwinia carotovora* subsp. *atroseptica* kunstmatig besmet. De kolonisatie-incidentie van *Erwinia carotovora* subsp. *carotovora* en *Pseudomonas marginalis* daalde tijdens de eerste weken van de bewaarperiode om na December significant toe te nemen tot het einde van de bewaarperiode in Maart. Dit patroon herhaalde zich in elk van de drie jaren van de bemonsteringsperiode. Tijdens de bewaarperiode in 1996 daalde het aantal *Erwinia carotovora* subsp. *atroseptica* populaties op witlofpennen tot medio November zonder daarna weer toe te nemen. Tijdreeks-analyse van de maandelijkse kolonisatie-incidentie van *Erwinia carotovora*

subsp. *carotovora* en *Pseudomonas marginalis* liet een afhankelijkheid tussen de incidentie en de incidentie twee weken eerder zien. Een hoge kolonisatie-incidentie tijdens de bewaarperiode ging ook samen met een hoge ziekte-incidentie tijdens de volgende trekperiode. Dit resultaat maakte het mogelijk om de ziekte-incidentie tijdens de trek van de witlofkroppen, gebaseerd op de kolonisatie-incidentie van de pennen tijdens de bewaring, te voorspellen.

Hoofdstuk 7 - Natrotbacteriën tijdens de trek

Witlof kan tijdens de laatste dagen van de trekperiode een slijmig rot ontwikkelen, die tot 60% uitval kan leiden. Het voorkomen en de aggregatie van de ziekte op witlofkroppen tijdens de trek en de invloed van teeltmaatregelen werd in een driejarig experiment onder twee trektemperaturen bestudeerd. De gemiddelde ziekte-incidentie was het laagst in 1994 en het hoogst in 1996. Zij bedroeg gemiddeld 0.07-0.13 in de vroege trekperiode en 0.15-0.18 in de twee latere trekperioden. Witlofkroppen die onder lage trektemperaturen getrokken werden en kroppen van pennen met een lage stikstofbemesting of van pennen gedompeld in calciumchloride vertoonden relatief weinig ziekte. De ziekte-incidentie was geaggregeerd en beta-binomiaal verdeeld. Chi-kwadraat analyse voor de goodness-of-fit was significant met $\theta \approx 0.15$ ($n = 136$, $p = 0.003$) voor de hoge temperatuur en met $\theta = 0.11$ ($n = 136$, $p = 0.016$) voor de lage temperatuur tijdens de trek. Het dompelen van witlofpennen in calciumchloride en lage stikstofbemesting tijdens de penproduktie verminderden de ziekte-incidentie en deden de aggregatieparameter θ toenemen. De ziekte vormde kleinere haarden in de trekbakken en verspreidde zich minder in de trekbakken.

Hoofdstuk 8 - Algemene discussie

Epifytische populaties van natrotbacteriën op witlofplanten kunnen natrot veroorzaken als bepaalde factoren ten gunste van de bacteriën veranderen. Onder andere de omgevingstemperatuur, eventuele fysiologische stress van de plant en het bereiken van het minimumaantal bacteriën nodig voor infectie zijn belangrijk. Tijdens de witlofproduktie treden er veranderingen op als het lof getrokken wordt. Water- en luchttemperatuur van de trekcellen bereiken waarden die voor de bacteriën geschikt zijn om zich te vermenigvuldigen. De bacteriën verspreiden zich

niet alleen via de waterfilm op de groeiende witlofkroppen maar kunnen ook door insekten overgebracht worden. Natrotbacteriën kunnen met behulp van fruitvliegjes en andere insekten wonden en groeischeurtjes bereiken, waar de bacteriën nieuwe infectiepoorten vinden. Daardoor kan zich natrot van de ene witlofkrop naar de andere verspreiden. Bestrijding van de ziekte is daarom maar op één manier mogelijk, namelijk door de oorzaken van de ziekte weg te nemen. Deze oorzaken zijn de vatbare fysiologische conditie van de plant en de voor de bacteriën gunstige omgevingsfactoren. Wanneer zowel de natuurlijke mechanische barrières van de kroppen tegen een bacteriële infectie versterkt worden alsook een voor bacteriën ongunstig milieu gecreëerd wordt, kan de intrinsieke resistentie van de witlofplanten de bacteriële groei en uiteindelijk de ontwikkeling van natrot tegengaan.

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Curriculum vitae

Brigitte Monika Schober werd geboren op 20 april 1966 in Salzburg, Oostenrijk. Zij behaalde haar eindexamen VWO (Matura) op het Bundesrealgymnasium in Innsbruck, Oostenrijk, met een exact vakkenpakket. Na haar verhuizing naar Nederland vervolgde zij haar studie in de biologie, vanaf 1988 in de planteziektenkunde aan de Landbouwuniversiteit. Van 1992 tot 1993 volgde ze twee afstudeervakken in de ecologische Fytopathologie in de onderzoeksgroepen van ir. T. Hijwegen en drs. G.J. Bollen. Daarnaast deed ze haar stage bij het Laboratorium voor Monoclonale Antistoffen te Wageningen. In november 1993 behaalde zij het ingenieursdiploma. Op 1 januari 1994 werd zij aangesteld als Assistent in Opleiding aan de vakgroep Fytopathologie om onderzoek te verrichten naar bacterieel natrot in witlof. Een groot deel van dit onderzoek is beschreven in dit proefschrift. Tijdens haar aanstellingsperiode was ze voorzitter van de AIO-raad van de C.T. de Wit Onderzoeksschool Productie Ecologie en organiseerde zij activiteiten voor deze onderzoeksschool.