Biocontrol of *Fusarium* in wheat – introducing bacteria to a system of complex interactions

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Abstract

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The rhizosphere microflora and its impact on plant growth is in many respects still unexplored, and biocontrol is part of a growing research area called 'plant-microbe interactions'. The aim of this study was to find bacterial isolates that were able to suppress wheat (*Triticum aestivum*) diseases caused by *Fusarium* spp. and *Microdochium nivale*. A special challenge was the snow mould disease, which brought about the low temperature approach used in this work. Psychrotrophic bacteria were isolated at +1.5°C and 598 isolates were screened in a field-correlated greenhouse bioassay, out of which 163 were tested for disease suppression under field conditions (**I**). In field experiments during five consecutive years biocontrol of wheat seedling blight, caused by *F. culmorum* and *M. nivale* was in many cases as effective as the fungicide (guazatine acetate) control. Three isolates suppressed snow mould in winter wheat and brought about an average yield increase of 1450 kg/ha (**I**). Of special interest was how selection during the isolation of bacteria could affect the proportion of isolates with disease suppressiveness (**II**). The isolates were grouped and analysed in search for isolation factors that had a propitious effect on the isolation frequency of such isolates. In total, 30 groups were defined. The colony morphology of the isolates, which is a crude reflection of bacterial species or group, was also analysed. An unexpected discovery was that there was a higher frequency of potential biocontrol agents isolated from plants of the Brassicaceae family. A morphological group containing 56 highly disease-suppressive *I*solates having characteristic *O*ptically *D*enser *S*pots in their colonies were found and denoted *IODS*. Members of this group have similar *in vitro* inhibition spectra of pathogens and produce an antifungal polyketide (DDR) (**II**, **III**). A second aim was to identify the mode of action of isolates with disease-suppressive ability. One of the IODS, strain MF 381 produced DDR, pyrrolnitrin, and a novel antibiotic named pseudotrienic acid A (**III**). In other strains, metabolites such as massetolide and viscosin were found (**II**). One of the strains (MF 30) had a high disease-suppressive effect against *F. oxysporum* f.sp. *radicis lycopersici* causing wilt in tomato. Results by other authors indicate that MF 30 induces plant resistance by means of bacterial lipopolysaccarides through the ISR pathway (**IV**; Konnova *et al*. 1999). Yet another mode of action is probably operating in the biocontrol of *Fusarium* spp. obtained with the non-antibiotic producing strain MF 626 (an atypical *Pantoea agglomerans*). A set of induced mutants with mutations in *in plantae*-induced genes has been created and are currently being characterised.

Keywords: BCA, field-correlation, low temperature, plant induced, *Pseudomonas* spp., rhizosphere, seed-applied, selective isolation

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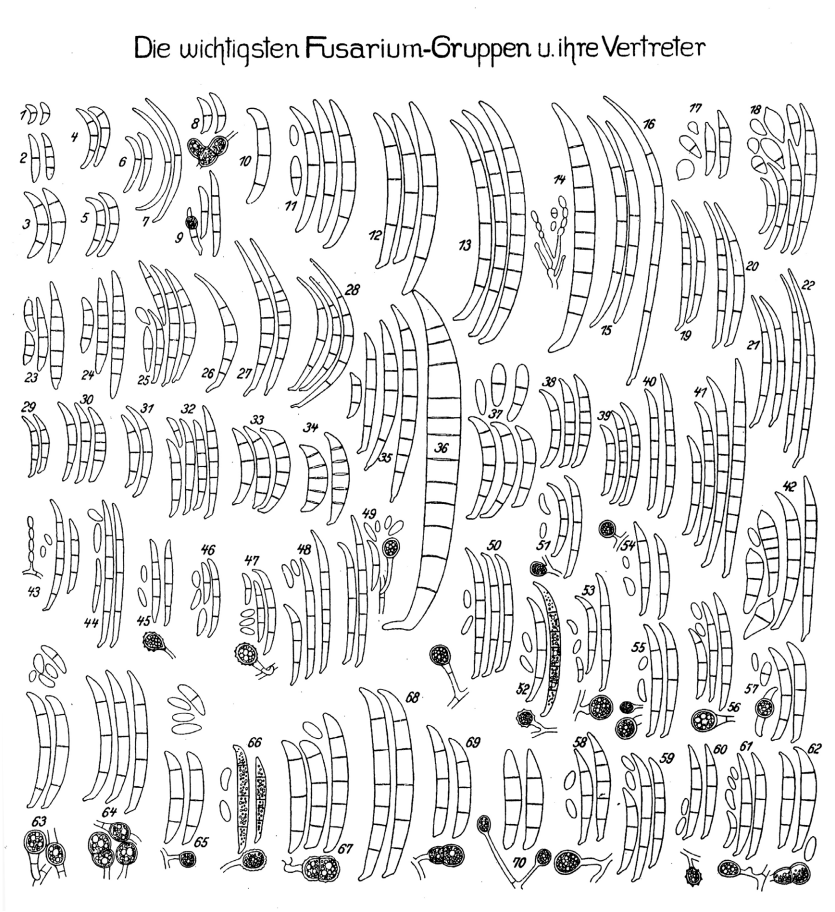
To Mamma who believed in me
- *and* to Iris who didn't.



This illustration was supposed to be on the cover, but it was 'forbidden' to have illustrations there, so you just have to imagine how it could have been.

"Neo, no one has ever done anything like this."
"That's why it's going to work."

Trinity and Neo in "The Matrix',
by the Wachowski brothers.

Preface

This project started with a passion for the beauty of the microscopic structures in fungi. A special challenge was the genus *Fusarium*. These organisms - mostly considered as disgusting moulds that destroy our crops and food and produce some of the most potent toxins known - are so beautiful! Their mycelia are often coloured in shades of red, orange, pink and covered with fluffy, white aerial mycelium. In the microscope, they have banana-shaped conidio-spores (see Figure 7 on page 48), and the sexual structures are small beakers that protect the spores. Anyway, there would barely be any funding for allowing a PhD-student sit all day and look in the microscope for beauty. When I got the opportunity to work with this group of "pathogens", with the aim of decreasing their destructive actions, I took it. My assignment was to search for potential biocontrol agents, BCAs, (see Glossary) by isolating and screening plant-associated bacteria. Now a completely new field of beauty was revealed, bacterial colonies are unexpectedly variable, in colour, in shape, and in structure! So after all, I was funded for sitting and looking in the microscope for beauty all days. In total, I observed and coded around 1000 bacterial isolates, and some of the results from this work are included in paper **II**. See also Figure 3 on page 36 and learn that bacteria can be so much more than some disgusting little things that barely have any right to exist. On the contrary, in this work their existence is not only justified, but new beneficial areas of their use are presented (**I** and **IV**). With accumulating knowledge of the rhizosphere environment, the idea comes to my mind – would plants be able to grow at all without them?

When I started, I had an idea that a Ph. D. dissertation should cover all knowledge in a certain area. That was perhaps possible some 100 years ago, but now, in the age of information technology, it is in most cases very difficult. The area of biocontrol, in which this thesis hopefully will be a contribution, has grown 'exponentially' and there are about 500 reports published yearly where the word 'biocontrol' is mentioned, so there is quite much that is known. In the literature review I have focused on subjects I find interesting and, in summary, the topics listed below have been treated.

- Mainly soil- and seed-borne fungi pathogenic to wheat, with special focus on *Fusarium* species infecting wheat, and *Microdochium nivale*.
- Ecology of culturable rhizosphere bacteria and the rhizosphere environment.
- Isolation of, and screening for root-associated bacteria with disease-suppressiveness.
- An overview of known mechanisms in disease-suppression.

In the last part of the literature review I have discussed some general thoughts that have been following me during this work, and that might be of interest for other people working with environmental microbiology. For instance: What is a bacterium – really?

Uppsala in April 2003 P.M.J.

Contents list

Appendix

Papers I - V

The present thesis is based on the following papers, which are referred to by their Roman numerals.

I. Johansson P.M., L. Johnsson & B. Gerhardson. 2003. Suppression of wheat-seedling diseases caused by *Fusarium culmorum* and *Microdochium nivale* using bacterial seed treatment. *Plant Pathology 52,* 219-227.

II. Johansson P.M. & S. A. I. Wright. 2003. Isolation factors affecting the frequency of disease-suppressive rhizobacteria and characterisation of a distinctive group of pseudomonads. Submitted for publication in *Applied and Environmental Microbiology.*.

III. Anton Pohanka, A., A. Broberg, L. Kenne, P.M. Johansson & J. Levenfors,. Antimicrobial metabolites from *Pseudomonas* sp. MF381-IODS, including a bioactive novel triene. Will be submitted for publication in *Journal of Natural Products.*

IV. Attitalla I., P.M. Johansson, S. Brishammar & B. Gerhardsson. 2001. *Pseudomonas* sp. strain MF 30 suppresses fusarium wilt of tomato *in vivo*. *Phytopathologia Mediterranea 40*, 234-239.

Papers I and IV are reproduced with the publisher's permission.

Glossary

Definitions on how I have been using the terms in the text. Some of the terms are also used for entomological applications, but for sake of simplicity that type of biocontrol is not included in the definitions.

Aggressiveness – The relative ability of a plant pathogen to colonize and cause damage to plants. The term is often used in epidemiology and describes differences among isolates of the same species.

Anamorph - The imperfect (asexual) stage of a fungus.

Antagonist – An organism that is able to suppress pathogenic fungi and bacteria in artificial systems, often detected as inhibition zones in dual culture plates (Figure 4, page 39). The term is often confused with biocontrol agent.

Antibiosis – A mode of action in biocontrol. The antagonist produces one or more substances that inhibits or kills the pathogen.

Biological control: (syn. biocontrol) – Exploitation by humans of natural competition, parasitism and/or antagonism of organisms for management of pests and pathogens

BCA – Biocontrol (Derived from **bio**logical pest **control**) agent –An organism that is able to suppress the undesired action of another (pathogenic) organism in different cropping systems. This term is also (mis)used for organisms that are able to suppress pathogenic fungi and bacteria in artificial systems. Some use the term only for organisms that have been developed into commercially available products.

DRB – Deleterious rhizobacteria – The term is used for bacteria that cause minor (or sometimes more) damage to the host plant, without any obvious symptoms, or without being classified as true pathogens by, for instance complying Koch's postulates.

Disease-suppression – The BCA is able to suppress disease development caused by the pathogen in the host plant, which can be achieved in various ways. It also involves the ability of the BCA to colonise the host (Figure 3 in **I**).

Dual culture (here) – A bacterial strain and a fungus are grown together on an agar plate and substances produced by the bacteria are able to diffuse through the agar and affect the fungus (Figure 4, page 39). This is a fast method to screen for bacteria that produce antibiotics and other substances that have a negative effect on pathogenic fungi. Also called *in vitro* antagonism/antibiosis.

Endophytic bacteria – Bacteria that are able to colonize the vascular bundle of the plant (inside the Casparian strip see Figure 2, page 26)

Ectorhizosphere – A thin soil layer that surrounds the roots and where the nutrition status due to leakage of root exudates and microbial activities is much higher than in the bulk soil.

Endorhizosphere – The epidermis and cortex cells of the roots and the intercellular area. The endorhizosphere is limited by the Casparian strip (see Figure 2) and any colonization interior to this limit is considered as endophytic.

Facultative parasite/Opportunistic pathogen – An organism that usually lives on decomposing dead material (saprotroph), but under certain conditions can turn pathogenic, e.g. if the host is stressed or weakened.

Fusarios(es) – Common name for all the various diseases caused by fungi in the genus *Fusarium*, and related fungi e.g. *Microdochium nivale* (syn. *Fusarium nivale*).

Generalist – An organism that is not specialised, that can use a broad spectrum of resources, and thereby has a broad niche.

Haustorium – Specialized branch of a parasite formed inside host cells to absorb nutrients.

Induced resistance – The defence system of the host is stimulated by inoculation of for instance a non-pathogenic relative to the pathogen or by another micro organism that has the ability to induce resistance in the plant.

in vitro – in glass, on artificial media, or in an artificial environment; outside the host.

in vivo – within a living organism, here sometimes used synonymously with *in situ* – in its original place or environment.

IODS – Isolates with Optically Denser Spots are a group of isolates that have high disease-suppressive ability and special colony morphology, which make them easy to recognise (see fig 1 in **II**).

Koch's postulates – The procedure used to prove the pathogenicity of an organism, and that the observed symptoms are caused by this organism.

Pathogenicity – Term that describes whether or not an organism is able to cause disease.

PGPR – Plant growth-promoting rhizobacteria – In this category BCAs are sometimes included. Other means of plant growth-promotion could be that the bacteria produce growth-stimulating hormones or increase the access of different inorganic substances e.g. by transforming them to more soluble forms or by changing the pH.

Pseudomonads – A group of bacteria that is commonly occurring many of which are well adapted to the rhizosphere environment. The group contains approximately 150 species but only a few have been used in biocontrol systems. The group is quite diverse and the taxonomy is constantly revised.

Psychrotrophic bacteria – Cold-tolerant bacteria with optimum growth at temperatures around 20-25°C that are able to be active also in temperatures close to 0° C.

Psychrophilic bacteria – Bacteria with growth optimum below 15° C.

Rhizosphere – The microenvironment in the soil immediately around, on, and to some extent in plant roots.

Rhizo(sphere)bacteria – Bacteria adapted to life in the rhizosphere, i.e. isolated from the rhizosphere.

Root exudates – The various compounds that leach from growing and expanding sections of roots as well as from broken cells at exit points of lateral roots. New research show that parts of the root exudates is actively secreted by the plant, which in this manner can communicate with the surrounding microflora.

Seed treatment – Application of a biological agent, chemical substance or physical treatment of seed, in order to protect the seed or plant from pathogens or to stimulate germination and plant growth.

Seed borne – Carried on or in a seed.

Virulence –The relative capacity of an organism to cause disease, or its ability to overcome the resistance of the host.

VA-mycorrhiza – Vesicular-Arbuscular mycorrhiza (VAM) – A symbiotic association between a non-pathogenic (or weakly pathogenic) fungus and the roots of a plant. In contrast to the ectomycorrhiza, VAM is mainly associated with herbaceous plants. Sometimes referred to as endomycorrhiza or just AM (Arbuscular mycorrhiza).

The following references were consulted while preparing the glossary:
http://www.apsnet.org/education/IllustratedGlossary/top.htm; Campbell, R. 1989. *Biological control of microbiological plant pathogens*. Cambridge university press. ISBN 0-521-34088-8. Pp. 184-191; Lawrence, E. 1990. Henderson's dictionary of biological terms. 10th edition. Longman Group Ltd., Essex, UK. ISBN 0-582-06433-3. 637pp.

Ordlista

Definitioner på hur jag har använt termerna i texten. Vissa av termerna används också för entomologisk biologisk bekämpning, t.ex. parasit-steklar och nematoder, men de är inte med i dessa definitioner.

Aggressivitet – En växtpatogens relativa förmåga att kolonisera och orsaka skada på grödan. Termen används ofta inom epidemiologin och beskriver skillnader mellan olika isolat av samma art.

Anamorf – Det asexuella stadiet av en svamp.

Antagonist – En organism som kan undertrycka patogena svampar och bakterier i artificiella system, ofta detekterat som inhibitions-zoner i tvåkulturs plattor. Termen är ofta sammanblandad med termen biokontroll agent.

Antibios – En av verkningsmekanismerna i biologisk bekämpning. Antagonisten producerar en eller fler substanser som inhiberar eller dödar patogenen.

Biologisk bekämpning: (syn. biokontroll) – Människans utnyttjande av naturliga processer som konkurrens, parasitism och/eller antagonism för att kontrollera sjukdomar och skadedjur.

BCA – *Biocontrol agent* (eng.) – En organism som kan motverka icke önskade effekter som orsakas av en annan (patogen) organism i olika odlingssystem. Termen är också (oegentligt) använd för organismer som kan undertrycka patogena svampar och bakterier i artificiella system. Vissa vill att termen endast ska omfatta organismer som blivit utvecklade till kommersiella produkter.

DRB – *Deleterious rhizobacteria* (eng.) – Skadliga rotbakterier – Termen används för bakterier som orsakar smärre (ibland mer) skada på värdväxten, utan att ge specifika symptom, eller utan att vara klassad som patogen t.ex. genom att uppfylla Kochs postulat.

Disease-suppression – Sjukdomshämmning – Biokontrollorganismen (BCA) har förmågan att hämma sjukdomsutveckling, orsakat av patogenen i värdväxten, vilket kan ske på olika sätt. Termen omfattar också BCA:s förmåga att kolonisera värdväxten (Figur 3 i **I**).

Dual culture (här) – Tvåkulturs platta. En bakterie och en svamp växer tillsammans på en agarplatta och ämnen som produceras av bakterien kan diffundera genom agarn och påverka svampen (se Figur 4 på sidan 39). Det är en snabb metod för att screena efter bakterier som kan producera antibiotika eller andra substanser som har en negativ effekt på patogena svampar. Kallas ibland för in vitro antagonism/antibios.

Ectorhizosfär – Ett tunt jordlager som omger rötterna och som har ett mycket högre näringsinnehåll än den omgivande jorden eftersom rötterna "läcker" näring (se rotexudat) och för att den mikrobiella aktiviteten är hög.

Endofytiska bakterier – Bakterier som har förmågan att kolonisera växtens kärlsystem (innanför det Caspariska bandet, se Figur 2 sid. 26)

Endorhizosfär – Utgörs av epidermis och rot cortex-celler samt utrymmet mellan dessa celler. Begränsas inåt av det Caspariska bandet (se Figur 2). All kolonisering innanför detta område anses som endofytisk.

Fakultativ parasit/Opportunistisk patogen – En organism som vanligtvis lever på att bryta ner dött material (saprotrof), men som under vissa omständigheter kan bli patogena, tex. om värden är stressad eller försvagad.

Fröbehandling – Tillförsel av t ex. en bakteriekultur eller en kemisk substans till utsädet (betning) för att skydda det mot sjukdom, öka grobarheten och stimulera tillväxten. I termen ingår också fysikaliska metoder t.ex. värmebehandling.

Fusarios(er) – Populärt samlingsnamn för alla olika sjukdomar som orsakas av svampar inom släktet *Fusarium*, och närstående arter som *Microdochium nivale* (syn. *Fusarium nivale*).

Generalist – En organism som inte är specialiserad, kan utnyttja ett brett spektrum av resurser, och har därmed en bred nisch.

Haustorium – Speciell struktur som bildas av parasiterande svampar inuti värdväxtens celler, som absorberar näring.

Inducerad resistens – Växtens försvarssystem aktiveras genom att man inokulerar med t.ex. en icke patogen släkting till patogenen, eller någon annan mikroorganism som har förmåga att inducera resistens hos växten.

in vitro – i glas, i ett artificiellt medium eller i en artificiell miljö; utanför värden.

in vivo – inuti en levande organism

IODS – "Isolates with Optically Denser Spots" är en grupp isolat som har hög sjukdomshämmande effekt och en speciell koloni morfologi som gör dem lätta att känna igen (se Figur 1 i **II**).

Kochs postulat – Metod som används för att påvisa om en organism är patogen och att de symptom man iakttar är orsakade av denna organism.

Patogenicitet – Term som beskriver om en organism kan orsaka sjukdom eller inte.

PGPR – Plant growth promoting rhizobacteria – Tillväxtstimulerande rhizobakterier – Till den här kategorin räknas ibland också BCA:s. Bakterier kan vara tillväxtstimulerande genom att producera växthormoner eller genom att öka tillgången på olika oorganiska substanser t.ex. genom att kemiskt förändra dem till mer lättlösliga former eller genom att förändra pH-värdet.

Pseudomonader – En grupp bakterier som är vanligt förekommande och innehåller många arter som är väl anpassade till miljön runt växternas rötter (rhizosfären). Gruppen innehåller cirka 150 arter men bara några få har använts som BCA:s. Gruppen är ganska heterogen och taxonomin revideras ständigt.

Psykrotrofa bakterier – Köldtoleranta bakterier som växer bäst runt 20-25° C men som kan vara aktiva också i temperaturer nära 0° C.

Psykrofila bakterier – Bakterier som har tillväxtoptimum under 15° C.

Rhizosfär – Mikromiljön i jorden omedelbart intill, på och till viss del inuti växtrötter.

Rhizo(sfär)bakterier – Bakterier som är anpassade till ett liv i rhizosfären.

Rotexudat – De olika ämnen som läcker ut från växande delar av rötter och från trasiga celler där sidorötter tränger fram. Ny forskning visar att en viss del av rotexudatet aktivt utsöndras av växten som på detta sätt kommunicerar med rotmikrofloran.

Utsädesburen – Smittan sprids via fröet.

Virulens – Den relativa förmågan hos en organism att orsaka sjukdom, eller förmågan att bryta värd(växt)ens försvar.

VA-mycorrhiza – Vesikulär arbuskulär mycorrhiza – En symbiotisk association mellan en icke patogen (eller svagt patogen) svamp och en växt. I motsats till ektomykorrhiza så är VAM oftast associerat till örtartade växter. Kallas ibland endomykorrhiza eller bara AM (Arbuskulär mykorrhiza).

Följande referenser konsulterades vid upprättandet av ordlistan:
http://www.apsnet.org/education/IllustratedGlossary/top.htm
Campbell, R. 1989. *Biological control of microbiological plant pathogens*. Cambridge university press. ISBN 0-521-34088-8. Pp. 184-191.
Lawrence, E. 1990. Henderson's dictionary of biological terms. 10th edition. Longman Group Ltd., Essex, UK. ISBN 0-582-06433-3. 637pp.

Svensk sammanfattning

I det här arbetet har egenskaperna hos köldtoleranta (psykrotrofa) rhizosfärbakterier studerats. Vid sex olika tillfällen isolerades rotassocierade bakterier med en metod som utvecklats på enheten för Växtpatologi och biologisk bekämpning. Totalt isolerades cirka 1000 bakterieisolat. Metoden utvecklades och varierades på olika sätt för att undersöka om det fanns faktorer som kunde öka frekvensen av bakterier med sjukdoms-hämmande effekt (**II**). Ett syfte med arbetet var att försöka hitta bakterieisolat som hade förmågan att hämma utveckling av snömögel i höstvete, som orsakas av *Microdochium nivale*. För att göra det utvecklades ett växthus-biotest som kunde användas för att screena igenom ett stort antal isolat. Bakterierna tillfördes systemet genom fröbehandling och såddes i småkrukor med osteril sandblandad torvjord. I testet ingick en köldbehandling direkt efter sådd (**I**). Mycket arbete ägnades åt att säkerställa att detta biotest hade "fältrelevans" dvs. att de resultat som iakttogs i testet också speglade de resultat som erhölls i fältförsök. Ett relativt stort antal bakterieisolat fälttestades och data användes för att etablera en korrelationskurva mellan fält och växthusresultat (**I**). En faktor som ansågs viktig för att uppnå denna korrelation var att använda en hög infektionsgrad i biotestet. Mycket tid ägnades därför åt att testa olika svampisolat och olika sätt att stimulera graden av infektion. Under detta arbete utvecklades också ett "sjukdoms- hämnings index" (disease-suppression index, DSI) som baserades på både uppkomst och symptom. Detta hade hög korrelation med friskvikten av plantorna, men var mycket snabbare att arbeta med (**I**). I den färdiga biotesten användes ett isolat av *Fusarium culmorum,* som har mycket hög virulens och orsakar grodd- fusarios, en sjukdom som utvecklas inom tre veckor efter sådd. För att få enhetliga resultat användes artificiellt infekterat utsäde och noggrann kontroll av temperaturen i växthuset. I biotestet testades cirka 600 isolat (**II**).

I fältförsök under fem säsonger testades totalt 134 bakterieisolat och av dessa valdes några få ut, som testades upprepade gånger. I fält testades effekter mot både groddfusarios och snömögel. Groddfusariosen kan också orsakas av *M. nivale* och detta testades med naturligt infekterat utsäde. Under sommaren 2000 testades fyra bakterieisolat för effekt mot fusarioser på tre olika platser i södra och mellersta Sverige. I dessa försök odlades vetet till skörd och effekter på både kvantitet och kvalitet mättes. Under vintersäsongen 2000-2001 upprepades dessa försök men på andra platser och med tre av isolaten. Resultaten av dessa försök gav att alla bakterieisolaten hade effekter likvärdiga med fungiciden som användes som kontroll. I försöket med höstvete som var utlagt i Hedemora var vädret gynnsamt för utveckling av snömögel och därifrån noterades också den högsta skördeökningen (bakterie behandlat jämfört med obehandlat, sjukt utsäde) som låg på 1643 kg/ha (**I**). Två viktiga slutsatser kan dras från detta arbete: dels verkar det lönа sig att lägga ner mycket arbete och omsorg på att se till att det man mäter på labbet och i växthuset också är det man kan förvänta sig att observera i fält; dels verkar det som att bakterieisolat som har hög förmåga att hämma sjukdomsutveckling i ett system också har denna effekt i helt andra system.

Möjligheten att hitta en selektiv metod att isolera bakterier med sjukdomshämmande effekt undersöktes i artikel **II**. Olika isoleringsmetoder jämfördes med

avseende på hur hög frekvens av sjukdomshämmande isolat som hittades. De c:a 600 isolaten som var screenade i biotestet hade fått ett index värde (DSI) mellan 0 och 12. Isolaten grupperades sedan på olika sätt och analyserades. För varje grupp räknades medel DSI ut, samt antalet isolat som var sjukdoms-hämmande, dvs. sannolikheten att hitta ett sjukdoms-hämmande isolat i den gruppen. En aspekt som analyserades var bakteriernas kolonimorfologi, vilket ger en grov uppskattning om art tillhörighet. Kolonimorfologin studerades i samband med isoleringen och olika karaktärer kodades i en matris så att isolat med liknande karaktärer snabbt kunde identifieras (se Figur 3 på sidan 36). De faktorer som analyserades var (antal grupper inom parentes): isoleringstillfällen (6), ursprungsland (2), effekt av förbehandlingar av växtmaterialet som användes för isolering (4), isolerings media (4), taxonomiska grupper i växtmaterialet (3) och kolonimorfologi: former och kanter (kolumn 1), färger (kolumn 2), textur (kolumn 3), övriga karaktärer (kolumn 4) och isolat med samma kod i alla kolumner (11 grupper totalt).

Resultatet av denna analys gav att isolat som var isolerade från växter inom familjen Brassicaceae (korsblommiga) i genomsnitt var mer sjukdomshämmande än de övriga. Det var också stora skillnader på resultaten från de olika isoleringstillfällena och isolaten från Schweiz hade högre, eller mycket högre medel DSI. Vid analysen av kolonimorfologin identifierades en grupp av 56 isolat där alla isolaten definierades som sjukdomshämmande. Dessa isolat har en gemensam karaktär som beskrevs som "Isolates with Optically Denser Spots (IODS)", prickiga isolat (till vardags). Tidigare har sex liknande isolat hittats på Enheten och dessa har också visat goda sjukdomshämmande egenskaper, där det mest kända isolatet är MA 342, som är den aktiva organismen i det kommersiella betmedlet Cedomon™. Det verkar alltså vara möjligt att designa selektiva metoder för isolering av denna typ av bakterier. Den viktigaste slutsatsen av detta arbete blir att det material och de metoder som används vid isoleringen av bakterier kan ha en stor betydelse för de resultat man senare får när isolaten screenas och fält-testas. En annan slutsats, som i mikrobiella sammanhang är mycket ovanlig, är att man på synliga karaktärer kan avgöra vilka egenskaper ett bakterieisolat har.

I samarbete med Institutionen för Kemi studerades ett antal bakterie isolat med avseende på produktion av antimikrobiella substanser (**III**). Isolat av olika arter som alla visat sjukdomshämning i växthus och fältförsök valdes ut. De isolat som skulle analyseras odlades i vätskekultur, centrifugerades med ultracentrifug för att separera celler och supernatant (vätskefasen). Supernatanten användes sedan för analysen i alla fall utom för MF 626 där också cellernas eventuella innehåll av antimikrobiella substanser analyserades. Supernatanten separerades ytterligare i en hydrofil och en hydrofob fas via en C18 kolumn, solid phase extraction (SPE). Dessa fraktioner testades för aktivitet, dels med växthusbiotestet, dels med en *in vitro* metod som detekterar hämning av sporgroning. Oftast detekterades högst aktivitet i den hydrofoba fraktionen. Denna separerades ytterligare med preparativ HPLC och aktiviteten detekterades. När enskilda toppar kunde identifieras med HPLC så analyserades dessa med masspektrometri och NMR för att fastställa strukturen på substanserna (se Figur 5). I flera av isolaten kunde man identifiera produktion av kända antimikrobiella substanser som 2,3-deepoxy-2,3-didehydro-rhizoxin (DDR), massetolid och pyrrolnitrin (Figur 5, sid. 41). De prickiga

isolaten som testades hade alla produktion av DDR, men prickarna består inte av denna substans eftersom mutanter som förlorat förmågan att producera DDR också har denna kolonimorfologi (**II**). I ett av isolaten, MF 381 (*Pseudomonas* sp.), hittades en helt ny substans som har antibakteriell effekt. Den nya substansen kallas pseudotriensyra A (**III**) (Figur 5).

Ett av målen med detta arbete var att utreda mer i detalj varför ett eller flera isolat har denna sjukdomshämmande effekt. För detta arbete valdes MF 626 (*Pantoea* sp.), ett isolat som har givit mycket goda resultat i fältförsök och som antagligen inte producerar några antimikrobiella substanser (**II**). För att undersöka om bakterien har gener som endast uttrycks *in vivo*, producerades mutanter där olika gener slumpmässigt slogs ut. För att selektera mutanter som var inducerade av plantan eller av svampen så användes en transposon utan promotor, vilket innebär att det främmande DNAt är beroende av bakteriens eget genregleringssystem för att komma till uttryck. Metoden med att göra mutanter för att förstå vilka gener som är "på" i olika situationer bygger på att man tillför en markör t.ex. antibiotikaresistens som uttrycks i stället för den gen som normalt finns på den platsen. Informationen finns dock kvar eftersom det tillförda DNAt har hoppat in utan att ta bort något. Eftersom man vet vilket DNA man har tillsatt så kan man lätt hitta det igen och då hittar man också genen som har blivit inducerad av plantan. När man väl har fått fram DNA sekvensen så kan man jämföra den med andra sekvenser som finns i databaser och på så sätt bilda sig en uppfattning om funktionen av det protein som genen kodar för. Vissa gener är alltid "på" och dessa var vi inte intresserade av. Dessutom slås alltid en del viktiga gener ut, t.ex. sådana som kodar för proteiner som behövs för att göra livsviktiga vitaminer eller sådana som har centralt reglerande funktioner, och dessa ville vi inte heller ha kvar. När de båda grupperna av mutanter var borttagna så återstod 80 mutanter, 40 som inducerats i närvaro av svamp patogenen och 40 som inducerats av växten. Mutanterna testades i växthusförsök och fyra av dem hade reducerad förmåga att hämma sjukdomsutveckling, medan en av dem var något effektivare än vildtypen. Dessa fem mutanter analyserades sedan ytterligare för att ta reda på vilka gener som hade slagits ut. Detta arbete är ännu inte avslutat. En annan mekanism som kan vara viktig i fallet MF 626 har observerats i mikroskopet (Figur 7 på sidan 48). På bilden kan man se att svampen har blivit deformerad och de enskilda hyferna (svamptrådar) är svullna och fulla med stora blåsor. Allt skräp som finns i bilden är antagligen svampens cellinnehåll som har läckt ut från hyfer som har gått sönder. Detta scenario observeras ofta när det är svampar som fungerar som BCA, och det kallas också mykoparasitism.

Arbetet som presenteras i artikel **IV** är mestadels utfört av förstaförfattaren till den artikeln, men det finns med i denna avhandling eftersom det belyser en viktig aspekt. I detta arbete screenades 50 av de isolat, som hela detta arbete är baserat på, för effekter mot en kärlpatogen, *Fusarium oxysporum* f. sp. *radicis-lycopersi*ci, som orsakar vissnesjuka i tomat. Det visade sig att MF 30 hade mycket god sjukdomshämmande effekt i detta system. Det är intressant eftersom detta isolat hade goda effekter i fältförsök, även i vintervete (**II**). Det har blivit demonstrerat förut att riktigt effektiva BCA har effekt mot många olika patogener oberoende av värdväxt. Arbetet med MF 30 har sedan fortsatt och det verkar som om detta isolat har förmåga att inducera resistens i tomatplantorna, dvs. sätta igång

växtens vilande försvarssystem, så att inte patogenen lyckas infektera. Man kan visa att det handlar om inducerad resistens genom att hålla isär patogenen och BCA, så att det inte finns möjlighet att BCA kan sända ut några antibiotika, eller konkurrera direkt med patogenen på något annat sätt. Ett annat sätt är att använda bakteriecellväggar och injicera dessa i växten. Man har nämligen visat att det är en viss del av bakteriernas cellväggar, lipopolysaccarider, fungerar som en signal till växten att sätta igång det inducerbara försvaret. I fallet med MF 30 så testades den sistnämnda metoden och det arbetet gjordes av Konnova (1999). Det visade sig att cellväggspreparationerna också hade förmåga att hämma vissnesjukan, så man kan dra slutsatsen att MF 30 kan inducera resistens i tomat.

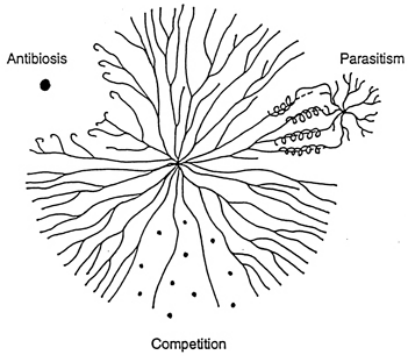


Illustration: Johan Schnürer

Microbial Antagonism against Fungi

Arbetet med denna avhandling har pågått under cirka sju år (deltid) och har varit finansierat av MISTRA (Miljöstrategiska fonden som upprättades från de gamla löntagarfonderna). Jag har ingått som en del av ett tvärvetenskapligt projekt där vårt huvudsyfte har varit att hitta ersättningar för kemiska bekämpningsmedel. Programmet kallas Microbial Antagonism Against Fungi (MAAF).

1. Introduction and aims of the study

1.1 Know your enemy

1.1.1 The Genus Fusarium

The fungi referred to as *Fusarium* spp. are the asexual state (anamorph) of several groups of Ascomycetes, and some of them have not yet been connected to their corresponding sexual state (teleomorph), (Boot, 1971; Nelson *et al.* 1983; Moss & Smith, 1984; Samson. & Reenen-Hoekstra, 1988). In the field of plant pathology this group of fungi are mostly known by their anamorph name and placed in the class Deuteromycotina (syn. Fungi imperfecti) (Nath, *et al.* 1970; Smith, 1981).

The group of fusarium pathogens and saprotrophes such as: *F. avenaceum, F. culmorum, F. equiseti, F. graminearum, F. oxysporum, F. poae, F. sporotrichoides, F. verticillioides* (syn. *F. moniliforme*) and the closely related *Microdochium nivale* (syn. *Fusarium nivale*), which are addressed in this work are well adapted to saprophytic growth and survival (Booth, 1971; Snijders, 1990). The literature in this area is quite confusing but it is clear that individual isolates of most of the above-mentioned species can cause a variety of different diseases (Garrett, 1970; Deacon, 1984; Bruehl, 1987). It is also clear that there is a high likelihood to isolate more than one of the species from plant tissue with disease symptoms. Hence it is not always easy to clarify the aetiology (Booth, 1971; Bateman, 1979; Smith, 1981; Moss & Smith, 1984; Windels & Holen, 1989; Parry, 1990; Cristani, 1992; Parry *et al.* 1995; Pettitt *et al*. 1996; Smiley & Patterson, 1996; Paveley *et al.* 1997; Schütze *et al.* 1997; Hare *et al.* 1999).

There can be as high a variation in virulence (aggressiveness) among isolates within a species, as among species (Windels & Holen, 1989; Miedaner *et al.* 1996; Miedaner & Schilling, 1996; Gang *et al.* 1998; Carter *et al.* 2002). Many isolates can be considered saprotrophes that are able to be pathogens, rather than pathogens *per se*. The fungi in the fusarium group are good competitors and they produce extra-cellular enzymes such as; β-glucosidase (Molot, 1967); cellulase, pectinase, and xylanase (Kang & Buchenauer, 2002; Wanjiru *et al.* 2002), and secondary metabolites such as mycotoxins (Vesonder *et al*. 1992; Toth *et al*. 1993; Perkowski *et al.* 1996; Langseth *et al.* 1997; Gang *et al*. 1998; Hörberg, 2001; Magan *et al.* 2002; Proctor *et al.* 2002, Table 1).

1.1.2 Fusarium species as pathogens - fusarioses

When acting as pathogens they mainly attack host plants that are; immature, e.g. seedling blight, root-, crown- and foot-rot (Pettitt *et al.* 1996); damaged, e.g. snow mould, leaf and stem infections (Parry *et al.* 1990); or senescent, e.g. node infection, or as in the case with scab (syn. head blight, ear blight) of wheat (*Triticum aestivum*) infection takes place at the stage of anthesis (at flowering when the pollen is mature) when the structures that will form the grain are tender and relatively unprotected (Parry *et al.* 1995). Most mentioned diseases are popularly referred to as fusarioses. The fusarium pathogens have no specialised structures for penetration of host cells, like appressoria or haustoria. If no wounds

are present, hyphae of *F. culmorum* are able to enter through the tips of lateral roots (Kamula *et al.* 1994), and enter the vascular bundle through cells in the Casparian strip, which are lacking suberin lamellae (Figure 2, page 26) Once inside the tissue, *F. culmorum* and *F. graminearum* are able to spread systematically (Snijders, 1990a; Clement & Parry, 1998; Kang & Buchenauer, 2002). When causing scab, *F. culmorum* infection occurs through the flowers. After building up a dense network of hyphae while degrading host cells through production of extracellular enzymes, the host cells are penetrated by means of a penetration peg (Kang & Buchenauer, 2002). Also, *F. graminearum* was observed inside the pericarp when causing scab, but when it was co-inoculated with an antagonistic *Streptomyces* species the fungal growth was limited to the outside (Fulgueira *et al.* 1996), indicating that the formation of the penetration peg never occurred. One strategy of the fusarium fungi to secure their position on the plants is to live epiphytically without causing symptoms (Clement & Parry, 1998). When the plant becomes senescent and wilts, they have a competitive advantage by already being established, when the saprophytic soil microflora get access to the nutrition source.

A special challenge in this work was to find bacterial isolates that could substitute chemical seed-treatment of winter wheat, with focus on the control of the snow mould pathogen *Microdochium nivale* (syn. *Fusarium nivale,* Samuels & Hallet, 1983) with the teleomorph *Monographella nivalis* (Muller, 1977). This fungus can be both seed-borne (Cristani, 1992) and soil-borne but is dependent on a snow cover on unfrozen ground to cause snow mould (Bruehl, 1987; Bruehl & Cunfer, 1971; Hömmö, 1994; Nakajima & Abe, 1994). In Scandinavia, as in many other temperate zones, the sowing of winter cereals is risky due to potential snow mould outbreaks, and in warmer regions this fungus is well known as one of the casual agents of scab. A questionnaire, made by Scottish Agricultural Science Agency (SASA), was sent to seed pathologists in ten European countries and a number of seed industry representatives. Respondents were asked to make an estimate as to which (maximum 3) seed-borne disease they considered to be of greatest economic importance. *M. nivale* was one of the pathogens that was mentioned as severe in all countries (Cockerell *et al*., 1997). Also in America, this fungus is a devastating and wide spread pathogen, together with other *Fusarium* spp. that are pathogenic to cereals, (Nakajima & Abe, 1994; Smiley & Patterson, 1996). The head blight (scab) disease, in particular, causes considerable losses. An estimated value of $3.5 billion (approx. 35 miljarder SEK) was lost in the USA and Canada, during the 1990s (Windels, 2000).

The production of mycotoxins (Table 1) is one of the factors which makes the head blight disease so serious, since it does not only decrease the yield, but also severely impairs the quality of the grain. It has been shown that some of the toxins, such as the trichothecenes are involved in the infection process (Bandurska *et al*. 1994; Miedaner & Perkowski, 1996; Bai *et al.* 2001; Kang & Buchenauer, 2002; Proctor *et al.* 2002). There is often a correlation between the levels of infection and the concentration of mycotoxins in the kernels (Perkowski *et al*. 1996; Evans *et al*. 1997; Gang *et al*. 1998), but when fungicides are used there can be a reduction in visible disease symptoms while the mycotoxin levels have increased (Magan *et al.* 2002).

Table 1: Mycotoxins produced by Fusarium species pathogenic to cereals

Species of *Fusarium*	Toxin	Crop[b]	References
F. avenaceum	Deoxynivalenol	Wheat	Toth *et al.* 1993
F. culmorum	Zearalenone	Wheat, maize,	Perkowski *et al.* 1996
	Deoxynivalenol	Wheat, rye	Toth *et al*. 1993; Gang *et al*. 1998
	Nivalenol	Wheat, rye, Barley	Gang *et al.* 1998; Perkowski *et al.* 1996 Hörberg, 2001
	Deoxynivalenoles[a]	Barley, wheat, oats	Perkowski *et al.* 1996; Hörberg, 2001 Magan *et al.* 2002
	Fusarenone	Wheat, oats	Hörberg, 2001; Magan *et al.* 2002
	HT-2 toxin	Wheat, barley	Hörberg, 2001
F. equiseti	Deoxynivalenol	Wheat	Toth *et al*. 1993
	Nivalenol	Wheat	Toth *et al*. 1993
	Zearalenone	Wheat	Toth *et al*. 1993
F. graminearum	Zearalenone	Wheat, maize	Magan *et al.* 2002
	Deoxynivalenol	Wheat	Toth *et al*. 1993; Magan *et al.* 2002
	Trichothecenes[c]	Wheat, maize	Proctor *et al.* 2002; Magan *et al.* 2002
	Fusarenone	Cereals	Magan *et al.* 2002
F. oxysporum	Moniliformin	Cereals	Magan *et al*. 2002
	Wortmannin	Cereals	Magan *et al.* 2002
	Fusaric acid	Cereals	Magan *et al*. 2002
F. poae	Trichothecenes	Cereals	Ohlsen 1996
	T-2 toxin	Cereals	Magan *et al.* 2002
	HT-2 toxin	Cereals	Magan *et al.* 2002
F. sporotrichoides	T-2 toxin	Wheat	Toth *et al*. 1993; Magan *et al*. 2002
	HT-2 toxin	Cereals	Magan *et al.* 2002
	Neosolaniol	Cereals	Magan *et al*. 2002
	Diacetoxyscirpinol	Cereals	Magan *et al.* 2002
	Fusarenone	Cereals	Magan *et al.* 2002
	Zearalenone	Cereals	Magan *et al*. 2002
F. verticillioides (syn. *F. moniliforme*)	Fumonisins	Maize	Proctor *et al.* 2002; Magan *et al.* 2002
	Moniliformin	Cereals	Vesonder *et al*. 1992; Magan *et al.* 2002
	Fusarin C	Cereals	Magan *et al.* 2002
	Fusaric acid		Vesonder *et al*. 1992

[a] 3-acetyldeoxynivalenol and 15-acetyldeoxynivalenol

[b] Maize (*Zea mays*), oats (*Avena sativa*), barley (*Hordeum vulgare*), rye (*Secale cereale*)

[c] Deoxynivalenol and nivalenol are examples of trichothecenes

1.1.3 Strategies to control diseases caused by Fusarium species

In Sweden, all certified winter wheat seed is treated with fungicides and one of the reasons is the risk of attacks by *M. nivale.* There are no fungicides that are really efficient against all the fusarioses, which under favorable conditions can attack the crop at different developmental stages (L. Johnsson, personal communication). Another problem with different fungicides that are applied by spraying at anthesis, is the risk of triggering an increase in mycotoxin production (Magan *et al.* 2002), hence a seed-treatment that has a prolonged effect would be valuable. The most common approach to deal with the fusarium diseases has been by breeding for resistant cultivars. The two diseases that has been the main focus in this context is snow mould (Gaudet, 1994; Nakajima & Abe, 1990; Hömmö, 1994; Nakajima & Abe, 1994; Mergoum *et al.* 1998) and scab (Snijders, 1990b; Bai, & Shaner, 1996; Miedaner & Perkowski, 1996; Miedaner, 1997; Hilton *et al.* 1998; Gilbert*,* 1998; Hilton *et al.* 1998; Mesterhazy & Bartok, 1998), but also crown/root rot (Waldermut *et al.* 1998) and seedling blight (Wisniewska & Chelkowski, 1996). Due to the complexity of the fusarioses, as mentioned in the first section, it is a difficult group to work with. An alternative strategy to control the fusarium pathogens is to store the seed at a high temperature (20°C) which can decrease the infection level (Gilbert *et al.* 1997), alternatively, to store the seed over a season (Bergstrom, 1993).

There have been relatively few attempts to find BCAs that are efficient against fusarium diseases in wheat, but there are some examples. Potential BCAs from the fungal kingdom have been used against *F. culmorum* (Birkedal Knudsen, 1994; Thavonen *et al.* 1994; Knudsen *et al.* 1995; Teperi *et al.* 1998: Davanlou *et al.* 1999). Bacteria have been tested against *F. culmorum* and *F. graminearum* causing scab (Kempf & Wolf, 1989; Fulgueira *et al.* 1996; Khan *et al.* 2001). Examples where seedling diseases caused by *Fusarium* spp. or *M. nivale* were suppressed by BCAs are: Kropp *et al.* (1996), Kim *et al.* (1997), Dal Bello *et al.* (2002), and this work (**I**). Huang & Wong (1998) obtained a reduction of crown rot by adding a BCA, *Burkholderia cepacia* (strain A3R) as soil drench. A different approach was used by Murray *et al.* (1986), who used purified gramicidin S (an antibiotic produced by *Bacillus brevis*) to inhibit spore germination of *M. nivale*. A lot of attempts have been made in trying to find effective BCAs of the wilt pathogen *Fusarium oxysporum* (various patovars) in crops other than wheat (Elmer, 1995; Wade, 1995; Leeman *et al.* 1996; Duffy & Defago, 1997; Chin-A-Woeng *et al.* 1998; Duijff *et al.* 1998; **IV**).

1.2 Aims and obstacles

The specific aims of this work were:

- To find naturally occurring microorganisms able to suppress diseases caused by fungi of the fusarium-complex.
- To find naturally occurring microorganisms able to suppress the snow mould disease caused by *Microdochium nivale*.
- To develop a field-correlated bioassay for screening of bacterial isolates.

- To design isolation methods with increased probability to find isolates with disease-suppressive effects.
- To do preliminary experiments in order to explain mode of action for one or few isolates.

1.2.1 Finding a test pathogen

In this work the bioassay, which was developed for the screening work, was built on seedling blight in winter wheat, caused by *Fusarium culmorum*, a disease that can be caused also by *M. nivale* (Paveley *et al.*, 1997; Hare *et al.* 1999). The seedling blight develops within 3 weeks after sowing giving clear symptoms (see fig 4 in **I**), which makes it is more suitable for a large scale screening than for instance the snow mould or scab diseases. The snow mould disease was very difficult to mimic, since it naturally develops under a snow cover, and the scab, which develops in the flowering plant would have been too time-consuming. It was not possible to work with *M. nivale* because there were no naturally infected seed lots available at the time. In order to get repeatable results, the ideal seed lot would have a high and even level of infestation. The use of artificial infestation with isolates of *M. nivale* was not feasible since the isolates tested lost their pathogenicity very quickly when cultivated on lab media. As reviewed above, many fusarioses can be caused by different species so, theoretically, bacterial isolates that are able to suppress one of them would also be able to suppress the others. That this supposition was correct has been shown in paper **I**, and it has also been demonstrated by other authors (Hoefnagels & Linderman, 1999).

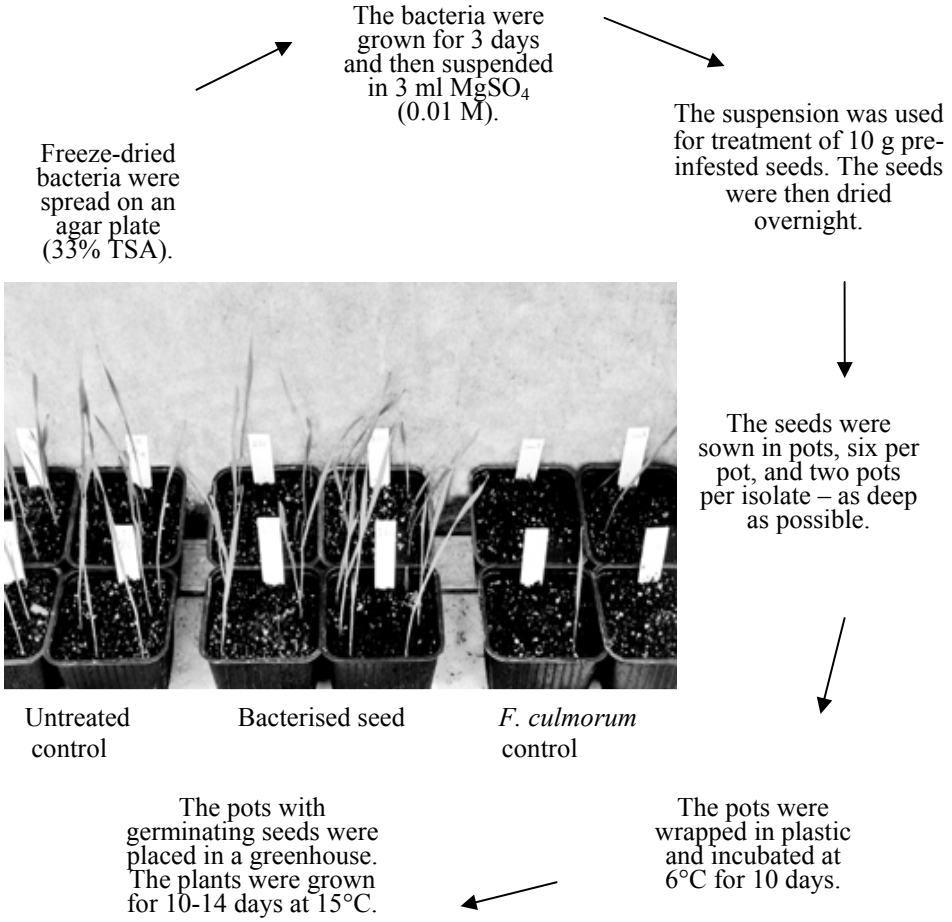
At the beginning, a lot of work was dedicated to isolating and testing different pathogenic isolates of *Fusarium* spp. and *M. nivale*. All isolates were obtained from seeds or other infected plant material, mainly from cereals, but also from plants of the Fabaceae family. After purification with methods described in Gams *et al.* (1988), the isolates were grown in Mung bean medium (Bai & Shaner, 1996) and their pathogenicity was tested in the greenhouse. The disease levels differed substantially among the isolates. Six *M. nivale* isolates had a disease incidence of 84%, 67%, 40%, 70%, 73% and 73% healthy plants, respectively, compared to 100% for the healthy control. Four isolates of *F. culmorum* were even more variable in virulence with a disease incidence of; 94%, 78%, 8%, and 5%, respectively. All other isolates showed no pathogenicity in this system, or weak pathogenicity, with the exception of one *F. tricinctum* isolate (57%). The most virulent *F. culmorum* isolate (No. 11), which allowed only 6% of the plants to remain healthy was chosen as the test pathogen for subsequent work.

1.2.2 Bioassay development

A basic concern when I developed the greenhouse bioassay was to secure high levels of pathogen infection. This was in accordance with recommendations in a mini-review where the experience from a joint Nordic programme of screening for BCAs is compiled (Knudsen *et al.* 1997). The idea behind this approach was the following; if the pathogen has optimal conditions, the selection towards isolates with a strong disease-suppressive effect will be pronounced, and thereby the chance to get isolates that will work well under field conditions should also

increase. Pilot trials aiming to enhance disease development included tests with soil-borne inoculum and a range of different time-periods of the low-temperature treatment as well as trials with different temperatures in the greenhouse. Some experiments with different covers on the soil were also performed. From these experiments, two factors appeared to be of importance: the temperature and the depth of sowing. The levels of infection decreased as the temperature increased, and at approx. 25°C no infection was observed. Initially, a type of low and wide pots was used where 50 seeds per pot were sown, a method developed at the Unit (Hökeberg, 1998), but these were changed to smaller and deeper pots. An advantage was that the increased sowing-depth resulted in longer coleoptiles (i.e. increased surface for infection). Another advantage was the time saved with only six seeds per pot to sow, which stimulated the use of more replicates instead. In the resulting assay the level of infection was high and repeatable (86% diseased and dead plants in average) and the temperatures used were 10 days at 6°C and 14 days at 15°C. The whole bioassay is represented by Figure 1.

Figure 1 The bioassay that was used when screening for potential BCAs



A 'Disease-suppressive-index' (DSI) was developed. The index measured both the emergence and the symptoms, and in (**I**) is described how it was assembled. The DSI was a good tool in the screening work as well as during the greenhouse checks of field-experiment material. The DSI correlated well with the fresh weight of the seedlings (**I**), which could be considered a more reliable but very time-consuming measurement of disease-suppression. A negative aspect of the use of the index was that it yielded discrete samples of data instead of continuous ones, which sometimes made the analysis more difficult.

In order to obtain a good correlation between field effects and results obtained under greenhouse conditions, isolation, bioassay-development, screenings and field-experiments were performed in parallel. Depending on the results, adjustments were done (see also 3.3). In the greenhouse screening for bacterial effects on *F. culmorum* infections, the pathogen infestation level and thereby, the level of disease was kept high. In the subsequent field experiments, the disease incidence was 47%, 5%, 28% and 29% with *M. nivale* as the causal agent of seedling blight or snow mould. The corresponding disease incidence with *F. culmorum* as the pathogen was 30%, 59% and 71% (**I**).

1.3 Biocontrol – manipulations of a system

Making a search for the term 'biocontrol' on the Internet gives some 30 000 hits, and in databases with scientific publications, for instance in BIOSIS 1989-2003 there were 6269 hits. So the subject is quite big and can not be covered by a single PhD-thesis, which raises the problem of limitation. Biocontrol is a system of many complex interactions involving Meteorology, Plant Physiology, Plant Pathology, Soil Sciences, Plant Nutrition, Microbiology, Biochemistry/Molecular biology and Chemistry, which makes it a challenging interdisciplinary area to work with. Different aspects are emphasised depending on the focus and interest of the person working with it.

My M.Sc. is in Biology, with Mycology and Plant Physiology and Systematics, rather than Agronomy as the main subjects, while the work that is presented in this thesis, was done at the Swedish University of Agriculture (SLU). Also, this work was done within the interdisciplinary project called 'Microbial Antagonism against Fungi' financed by the Swedish Foundation for Strategic Environmental Research (MISTRA), where people from three biological departments and the Department of Chemistry were involved. This is reflected by the somewhat different themes of the papers. Paper **I** can be defined as a contribution to traditional Plant Pathology being a subject within Agronomy. In paper **II,** the perspective is more Environmental Microbiology with a proportion of Systematics as a subject within Biology, and paper **III** is pure Chemistry. Paper **IV** is a Plant Pathology paper but the mechanism of induced resistance is also Plant Physiology. Finally the ongoing work with MF 626 could be defined as Molecular Biology mixed with Plant Physiology. Everyone understands that the consequence is that my knowledge of each of these areas can not be expected to be very profound, but instead I feel that it has given me a good possibility to have a 'system thinking', when I try to understand what is going on 'down there'.

In this introduction I would also like to refer to some selected works by authors with somewhat different perspectives on biocontrol in a broader sense, which I think can serve as a good basis for an introduction to this exciting subject. Already in 1952 scientists discussed many of the issues we still are discussing, such as the role of antibiotics in soil, the species composition of the rhizosphere microflora, and how this varies with plant species and varieties (Lochhead, 1952). Also, the complexity of this special environment and the limitations of different methods, especially regarding the ability to cultivate bacteria, was early realised (Foster, 1983). The practical/commercial obstacles were pointed out by Weller (1988) in one of the most cited reviews in this area. Other reports with a practical orientation are: Knudsen *et al.* 1997; Johnsson *et al.* 1998; Hoitink & Beohm, 1999, while the following papers discuss mechanisms on the molecular level; and ecological aspects of plant-microbe interactions in general (Rainey, 1999; Lugtenberg *et al.* 2001; Kent & Triplett, 2002). Yet another perspective, the chemical, are given in Leisinger & Margraff (1979) and Dowling & O´Gara, (1994). Books and book chapters that give a good orientation of the variety of mechanisms and interactions that are involved in biocontrol are; (Campbell, 1989; Young & Burns, 1993; Handelsman & Stabb, 1996; van Loon *et al*. 1998; Glick *et al.* 1999; Gerhardson & Wright, 2001). Finally, I would like to recommend some recent papers that have inspired me: (Shapiro, 1998; Ellis *et al.* 2000; Hawes *et al.* 2000; Whipps 2001; Berg *et al.* 2002).

2. Ecology of soil and root inhabiting bacteria

2.1 The rhizosphere

The rhizosphere as defined by Lynch (1982) consists of the plant roots and the soil layer in their proximity. The rhizosphere is further divided into the rhizosphere soil (ectorhizosphere), the root surface (rhizoplane) and the root epidermis and cortex intercellular space (endorhizosphere). Bacteria that are able to colonize beyond the Casparian band are normally referred to as endophytes (see Fig 2) (McInroy & Kloepper, 1995). In this special environment where the plant is the provider of nutrition many different life forms are competing for their life space, and our knowledge concerning the dynamics in this system is quite limited (Young & Burns, 1993; Grayston *et al.* 1996; Rainey, 1999; Kaiser *et al.* 2001). This area has earlier not been possible to study in much detail since most organisms associated with roots have not been possible to cultivate in the lab (Foster, 1983; Torsvik *et al.* 1990; Kent & Triplett, 2002). But in recent time the understanding has increased. With molecular methods such as, repPCR (Schneider & De Bruijn, 1996); DGGE (Normander & Prosser, 2000; Smalla *et al.* 2001); IVET (Rainey, 1999); gfp (Gage *et al.* 1996; Tombolini *et al.* 1999); and many more, it is now possible to study the dynamics in the rhizosphere without the limitations imposed through isolation and cultivation on laboratory media.

2.1.1 Root exudates and root border cells

It is known that the concentration of sugars, amino acids and other carbon sources, so called root exudates, are much higher in the rhizosphere than in the surrounding soil (Garett, 1970; Hale *et al.* 1978; Foster, 1983; Grayston *et al.* 1996), which in turn affects the concentration and type of microorganisms found (Germida *et al.* 1998; Miethling *et al.* 1999; Marchener *et al.* 2001; Smalla *et al.* 2001; Whipps, 2001; Berg *et al.*, 2002). In addition the composition and quantity of the root exudates varies considerably among different plant species (Garett, 1970; Hale *et al.* 1978; Grayston *et al.* 1996). Usually the plant-derived organic substances are distinguished in four types; exudates and lysate (including cell wall components) that are passively released, and secretions and mucilages, which are actively transported out from the roots (Foster, 1983; Lynch, 1990). There is surprisingly much of the plants' assimilation that leaks out into the surrounding soil. It is estimated that 10-40% of the carbon is deposited in the soil, rather than incorporated in the biomass of the plant (Grayston *et al.* 1996). Parts of this is due to the respiration of the plant roots, and depending on the method used, different researchers reach quite different conclusions concerning the proportion that should be defined as root exudates (references in Hale *et al.* 1978, and Grayston *et al.* 1996). Exudations are greatest at the root tip and by the elongation zone (see Figure 2). At the root tip the root cap is constantly breaking down, (see also below about root border cells), and at the elongation zone the fast turnover of the root hair cells considerably contribute to the exudates (Foster, 1983). The organic compounds that are released range from simple sugars, to enzymes and growth regulators. In work by Graystone *et al.* (1996), around 80 different substances are listed, but those frequently reported are: sugars, amino acids and organic acids (Hale *et al.* 1978, Grayston *et al.* 1996).

The microflora present on the roots can influence the amount of root exudates. Comparisons of the amounts of carbon released in presence and absence of root microflora showed that twice as much carbon was "lost" in the former situation (Lynch, 1982). Parts of the increased "surface-deposition" of carbon can be due to the microbial consumption of the mucilage layer surrounding the roots (Foster, 1983), and the replacement by the plant of this protective layer, with new polysaccharides. Many rhizobacteria do also form slimes and capsules, which merge with the plant-secreted mucilage especially on older parts of roots, and this matrix is called mucigel (Jenny and Grossenbacher, 1963). A known mechanism of pathogenesis of weak pathogens called deleterious rhizobacteria (DRB) is to increase the amount of exudates by producing HCN (Åström, 1991). Interestingly, this is also a biocontrol mechanism known for several BCAs (Voisard *et al.* 1989; Ellis *et al.* 2000). The group of bacteria referred to as plant growth-promoting rhizobacteria (PGPR) can influence the nutritional status in the rhizosphere in several ways. Many PGPRs are able to produce plant hormones, such as cytokinins, auxins, and gibberellins (Brandl & Lindow, 1997; Glick, 1999; Brandl *et al.* 2001). Alternatively, the PGPR can stimulate or suppress the plants' own hormone production. For instance, it has been shown that some strains have an enzyme that cleaves the plant ethylene precursor ACC (1-aminocyclopropane-1-carboxylate) (Glick, 1999). The growth-promoting effect of some PGPR is attributed to changes of the abiotic environment by an increase/decrease of the pH

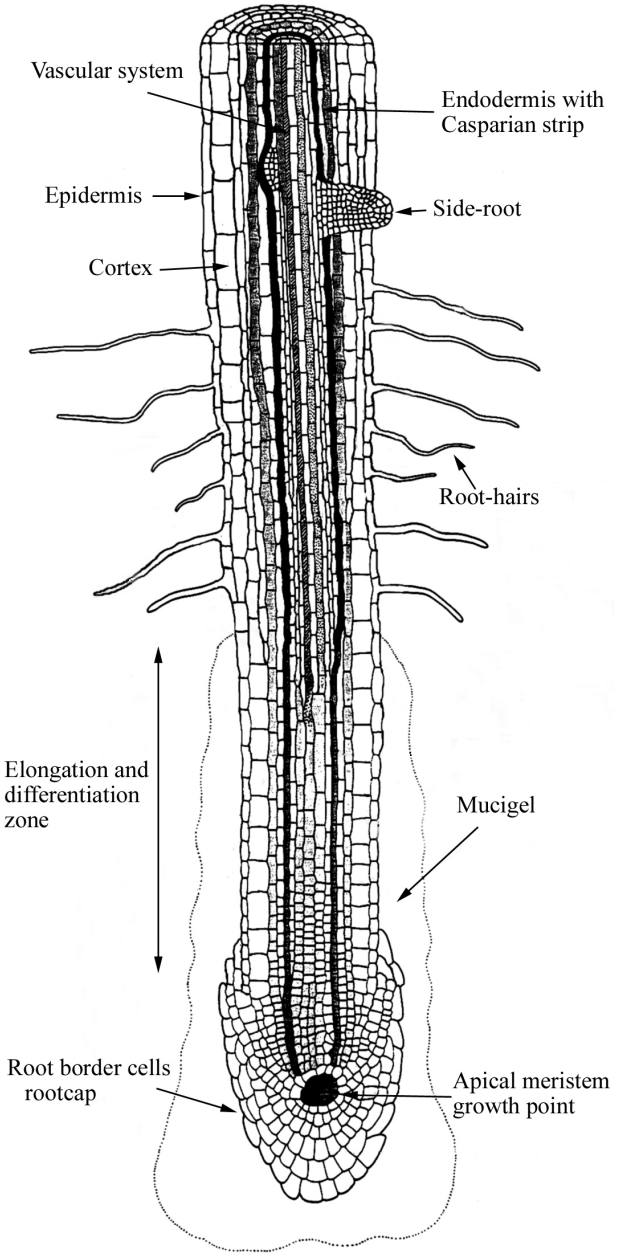
or mineralization of nutrients by means of extracellular enzymes (Glick, 1999). Yet another mechanism of growth promotion can be that the PGPRs interact with the native microflora, e.g. by suppressing the action of DRBs (Kloepper & Schroth, 1981).

There is also evidence of how the plant itself actively can influence the composition of the nutrition status in the rhizosphere. Hawes *et al.* (1991) demonstrate that plant roots under certain conditions can initiate the production of so-called border cells (see Figure 2). In situations where free water reaches these cells (e.g. after a rain), they are released from the root tip and can live as unicellular organisms around the root for up to a week in field soil, or several months in hydrophonic cultures (Hawes *et al.* 2000). The release of the BC is a rapid process where one layer of cells is completely separated within one hour. The gene expression of the border cells is distinct from the other root cells, and especially extracellular proteins are released in more than ten times higher quantities than from other root cells. The border cells can also function as reservoirs for chemo-attractants, and these might respond to specific signals from pathogens in order to draw attention from the root tip itself (Hawes *et al.* 2000). This mechanism works with root knot nematodes, which are attracted to the border cells and due to their secretion of an unknown substance the nematodes get immobilized for several hours (or days, depending on the assay used). Similarly, pathogenic fungi and bacteria confusedly attach to the root border cells, rather than to the rot tip (Gunawardena & Hawes, 2002). These researchers have also shown that there might be a correlation between the production of border cells, which differs among plant species, and the formation of VA-mycorrhiza (Niemera *et al*. 1996).

2.1.2 Symbiotic associations in the rhizosphere

The most studied symbionts in annual crops, of the temperate zone, are the nodule-forming *Rhizobium* spp. associated mainly with plant species in the Fabaceae family (Campbell, 1990; Prescott *et al*. 1993; Gage *et al.* 1996). When establishing this association a specific communication between the host plant and the free living bacterial cells occurs. Another wide-spread symbiotic relationship is the Zygomycetes of the order Glomales, which associate with 80% of all plant species on earth forming VA-mycorrhizas (or endomycorrhiza). These fungi are obligate biotrophs and survive as zygospores (sexually produced resting spores) when the host is absent. This association might be crucial to plant growth, especially in terms of uptake of phosphorous compounds (Kling, 1993; Prescott *et al*. 1993; Niemira *et al.* 1996). Their establishment is less specific than the association process of legumes and rhizobia, but it has been shown that their spore germination is stimulated by carbon dioxide and plant secreted flavonoids (Tsai & Phillips, 1991). For unknown reasons, plants of the Brassicaceae and Chenopodiaceae families do not have this type of symbionts (Kling, 1993; Niemira *et al.* 1996). To further illustrate the complexity of the different interactions in the rhizosphere, a VA-mycorrhiza-forming fungus, *Gigaspora marginata*, was found to have a bacterial endosymbiont of the genus *Burkholderia* (Ruiz-Lozano & Bonfante, 2000). The authors identified the presence of a gene (*vacB*) in the endosymbiont, which is involved in colonisation of host cells.

Figure 2 Longitudinal cross section of a root (adapted after Taiz & Zieger 1991)



In my opinion, it seems unlikely that not also other such specific and beneficial associations occur between plants and microorganisms. There are some reports that indicate that the plants in fact selectively can facilitate the colonisation of

certain groups of microorganisms. Tepletski *et al.* (2000) found that many plant species can produce an *N*-acyl homoserine lactone-(AHL)-mimicking signal. They also confirmed that this signal had differential effects on different types of bacteria. The AHL-signal is frequently used by Gram-negative bacteria to regulate density-dependent gene-expression (so called quorum sensing (QS), Swift *et al.* 1996), and it has been shown to be involved in the regulation of the production of some metabolites that are involved in biocontrol (Pierson III, *et al.* 1994; Farrand *et al*. 1996). In fact, Tepletski *et al.* (2000), included a biocontrol strain, *Pseudomonas aureofaciens* 30-84, active against *Gaeumannomyces graminis* in wheat (Pierson III & Thomashow, 1992; Scott, *et al.* 2002), as one of the AHL-induced reporter strains, but in their system this strain was not induced by the plant-produced AHL-like signal. Interestingly, two of the tested plant species did not produce the AHL-like signal: Lettuce (fam. Asteraceae) and *Arabidopsis thaliana* (fam. Brassicaceae), indicating that the signal might originate from root border cells (see above). Rainey (1999) characterized a number of genes that were induced *in vivo* in a PGPR, *P. fluorescens* strain SBW25. One of the genes had similarities to known pathogenicity genes of the Type III secretion pathway, which are involved in the specific recognition between plants and pathogens during pathogenesis. Continued work concluded that the Type III components found in this strain were different from those in phytopathogenic bacteria. Also, the presence of these genes was confirmed in nine other strains so they seem to be common in certain groups of bacteria (Preston *et al.* 2001). The Type III secretion pathway genes have also been identified in the symbiotic nodule-forming rhizobia (Marie *et al.* 2001).

The contribution of the plant to beneficial associations with microorganisms has also been studied. Plant genes that somehow increased the positive effect of microbial inoculation were identified as early as 1975 (Atkinson *et al.* 1975), but then it seems that the focus was changed towards the pathogen – BCA interaction and the role of the plant was less studied. In recent times though, this aspect of biocontrol has attracted renewed interest. In a study by Smith *et al*. (1999) three loci for quantitative traits were identified. They studied the variation in these loci with respect to disease control of *Pythium torulosum* by a PGPR, *Bacillus cereus*; strain UW25, using 61 recombinant inbred tomato lines. Two of the loci were associated with increased growth of the PGPR, and one was not. The interpretation by the authors was that the loci which did not enhance increased bacterial population sizes might have stimulated higher production of some disease-suppressive factors in the bacteria. The three loci identified in this work were of a quantitative nature; hence it might be less likely that they are involved in a specific communication between the plant and the microorganisms. There are some reports where it has been shown that the expression of plant-beneficial bacterial genes are actually induced by, or regulated by the plant (Brandl & Lindow, 1997; Brandl *et al.* 2001). The mentioned studies and other similar studies (Atkinson *et al.* 1975), are a reminder of the presence of the third actor in the biocontrol drama – finally entering the stage. In many reports, the plant is regarded as a passive component that secretes different substances, even though the above-mentioned reports and the work by Hawes and co-workers on the root border cells illustrate that the plant indeed is not passive.

In this work, one of the 33 strains that had full disease-suppression (**II**) was chosen for further studies on possible mechanisms involved in biocontrol. The chosen strain is an atypical *Pantoea agglomerans*, strain MF 626. This strain had high disease suppressiveness in field experiments and almost no *in vitro* inhibition of pathogenic fungi and bacteria (**I** & **II**, see also *4.3.3*). A working hypothesis was that specific genes in MF 626 were induced by the plant and/or the pathogen (*F. culmorum*), or both *in vivo*. To test this, a set of promoterless mini-Tn*5CatuidA* mutants were constructed by conjugation with SM10λpir carrying a plasmid with this transposon. The inserted transposon had also a constitutively expressed kanamycin (Km) resistance, while the wildtype of strain MF 626 is Km-sensitive. The mixed mutants were suspended in $MgSO_4$ (0.01M) and used to coat seeds of healthy and *F. culmorum*-infected wheat. The dose was equal to the one normally used in the bioassay (300 ml/kg, and a cell concentration of approx. 10^9-10^{10} cells/ml). The seeds were then sown in unsterile peat/sand soil and incubated at 6°C for different periods of time before being challenged with a high concentration of chloramphenicol (Cm, 200 ppm). Mutants with insertions in constitutively expressed genes were excluded as well as mutants that were unable to grow on minimal medium (MM) (auxotrophs). That is, constitutive mutants were excluded by only saving those that grew on MM and not on rich medium supplemented with Cm. These mutants were then tested with the greenhouse bioassay. Of each of the two situations (with and without *F. culmorum*) 40 mutants were saved and screened for disease-suppressiveness. This work is not yet finished.

2.1.3 Plant species-dependent variation of microbial communities

The bacteria that colonize the ecological niche of plant roots and the soil in their immediate vicinity are referred to as rhizosphere bacteria, or rhizobacteria. Several reports indicate that the microflora in the rhizosphere is quite distinct from that in the bulk soil (Foster, 1993; Westower *et al.* 1997; Germida *et al.* 1998; Smalla *et al.* 2001; Berg *et al.* 2002). Germida *et al.* (1998) concluded that the bacteria in the ectorhizosphere are mainly of soil origin, while the bacteria of the rhizoplane and the endorhizosphere are of seed origin, while Normander & Prosser (2000) stated that rhizoplane bacteria had more similarity to the soil bacteria in DGGE-profiles.

It is also well-documented that the composition of the microflora and the ability of introduced strains to colonise vary with the plant species (Foster, 1993; Glandorf *et al.* 1993; Lemanceau *et al.* 1995; van Overbeek & Van Elsas, 1995; Westower *et al.* 1997; Germida *et al.* 1998; Smith & Goodman, 1999; Remus *et al.* 2000; Smalla *et al.* 2001; Berg *et al.* 2002; Kent & Triplett 2002). Moreover, it seems as if these plant species dependent differences also affect the number of potential BCAs. In **II** we found a plant species dependent difference in the frequency of disease-suppressive isolates, with almost twice as high a frequency obtained from members of the Brassicaceae family (seven species) than from the other groups. Berg *et al.* (2002) found more bacterial isolates antagonistic to *Verticillium dahliae*, *Rhizoctonia solani, Sclerotinia sclerotiorum* and *Pythium cactorum* on strawberry roots than on oilseed rape and potato. From strawberry, the antagonistic isolates were mainly *Pseudomonas putida*, while a greater variety

of such isolates was found in oilseed rape. Also, Germida *et al.* (1998) found a more diverse flora of endophytic and rhizoplane isolates in oil seed rape than in wheat. Lynch & Panting (1980) analysed soils from two adjacent plots: one with grassland and one where oil-seed rape was cultivated. They found about three times more microbial biomass in the grassland than in the arable land, but the root biomass was almost 30 times higher in the grassland. Hence, the dry-weight (w) ratio w_{micobe} / w_{root} in oilseed rape was nine, compared to grassland where it was one. In a study of the root microflora of oilseed rape Kaiser *et al.* (2001) identified 214 different 16S rDNA sequences distributed over 32 bacterial genera.

It is possible that the microflora of Cruciferous (popular for fam. Brassicaceae) plants is generally more variable than, for instance, that of grasses. From a botanical point of view, it is not so surprising that the Brassicaceae family turned out to be special in this context. Like the Poaceae, Fabaceae and Orchidaceae families, this group of plants is big (contains many species), cosmopolitic and highly specialized, traits that are characteristic for what in botany is called a climax-group (Hultgård & Jonsell, 1988). The Fabaceae plants have their well-known association with bacteria (Gage *et al.* 1996), the grasses have the VA-mycorrhizas (Kling, 1993; Niemira *et al.* 1996), and orchids their particular type of mycorrhiza (Campbell, 1990; Zettler & Hofer, 1998). Why should not also the Crucifers have something similar, only less apparent?

2.2 What is a BCA?

In this work bacterial isolates were found that had disease-suppressive effects against seedling diseases in wheat, caused by fungi in the fusarium complex (**I**). Of the almost 600 isolates that were tested with the bioassay, 33 completely suppressed disease development (**II**). Those 33 isolates could of course answer the question above; because I think most will agree that they can be called BCAs, but what are they in their natural environment, these strains we call BCAs? Are there some characters that only can be found among the BCAs, or are they just randomly distributed bacterial strains that by chance are found? I think many researchers before me have asked these questions (for instance Ellis *et al.* 2000) and so far I do not think we have any satisfying answers to them. It is perhaps easier to tell what a BCA not is.

Among the 33 isolates that totally suppressed seedling diseases in wheat, only one was isolated from wheat. Most researchers have used the (most logical?) approach to isolate potential BCAs from the environment where they are intended to be used, but there are also examples where the plant species used for isolation was another than the crop where the BCA subsequently was used (Sun *et al*. 1995; Hökeberg *et al.* 1997; Johnsson *et al*. 1998; this work **I** & **II**). More often, a BCA that has effect in one system has been tried also in other hosts, often with as good effect (se below). The above-mentioned indicate that a BCA is not highly specialized to live in association with a specific host plant.

It is known that many BCAs are able to suppress various diseases in different host plants, for instance *P. fluorescens*, CHAO that was isolated from a soil suppressive to black root rot of tobacco (Voisard *et al.* 1989) and in addition to

this disease, CHAO also suppresses *Pythium* damping off in Cress and Cucumber (Maurhofer *et al.* 1994); crown and root rot caused by *F. oxysporum* f.sp. *radicis-lycopersici*, in tomato (Duffy & Défago, 1997); as well as a number of soilborne diseases in wheat (Défago *et al.* 1990a & 1990b). Likewise, MF 30 (atypical *P. veronii*), (**II**) initially isolated from a *Rumex acetocella* plant, has suppressed disease development of; *F. culmorum* and *M. nivale* in wheat (**I**); *F. oxysporum* f.sp *lycopersici* in tomato (**IV**);´ and *D. teres* in barley (Konnova *et al.* 1999). Another example of a BCA that has demonstrated activity in more than one system is *P. fluorescens*WCS417, which suppresses *Gaeumannomyces graminis* in wheat, fusarium wilt in carnation and radish, and had growth promoting effect in tomato by suppression of DRBs (Leeman *et al*. 1995). By judging from these examples a BCA does not seem to be disease-suppressive in a specialised manner, rather does a typical BCA have a high potential to adapt to different conditions.

There are some reports that strains with biocontrol activity have ability to use carbon sources that many bacteria are unable to use. For instance, tartaric acid (Khan *et al.* 2001), adonitol, cellobiose, D–malate, L–fucose, maltose, raffinose (Table 5 in **II**), malic acid, succinic acid and citric acid (Lugtenberg *et al.* 2001), linoleic acid (van Dijk & Nelson, 1998), inositol, saccharose, erythriol, m-hydroxybenzoate and 5-ketogluconate (Lemanceau, *et al.* 1995), organic acids (Goddard et al. 2001) are carbon sources that are utilised exclusively/preferentially by some groups of bacteria. So far, the picture of a typical generalist appears. That is, a bacterium with high ability to adapt to various nutritional supplies, and to various other factors like temperature and soil type, which were quite dissimilar when growing for instance tomato, compared to when growing wheat (**I** & **IV**).

A number of different mechanisms have been connected with biocontrol and in Chapter 4 some of these will be discussed in more detail. Many BCAs have production of so called secondary metabolites, especially the fluorescent pseudomonads are able to produce a variety of substances of which many are antibiotic (Leisinger & Margraff, 1979; Thomashow *et al.* 1990; Vincent *et al.* 1991; Pfender *et al*. 1993; Pierson III *et al.* 1994; Kraus & Loper, 1995; Hökeberg, 1998; Thomashow & Weller, 1998; Voisard *et al.* 1989; Wright *et al.* 2001). Also production of extracellular enzymes is a common feature among BCAs (O'Sullivan *et al.* 1991; Friedlender *et al.* 1993; Sacherer *et al.* 1994; Chernin *et al.* 1995; Frändberg, 1997; Dunne *et al.* 1997; Borowicz, 1998; Neiendam Nielsen *et al.* 1998; Berg *et al.* 2001), as well as iron chelating compounds like siderophores, (Defago & Haas, 1990; Thomashow & Weller, 1990 & 1996). The above listed references further strengthen the conclusion that a BCA is an organism that seems very well prepared to meet obstacles of different kinds and benefit from them.

In paper **II** we report that disease-suppressive bacteria were more often isolated from plants belonging to the Brassicaceae family. This finding can seem a bit contradictory to the conclusions drawn above, but it is not necessarily so. In the previous section the plant species-dependent variation of microbial communities was discussed, and there were several reports of a relatively high variation of bacteria found on the roots of Crucifers. A phenomenon known from botany is that in habitats where the conditions are hard, the variability is high. One would

expect it to be the other way around, but the reason is that the fast growing species out-compete all others in places with abundant nutrition and even water supply. In a dry pasture, on the other hand, the flora is very rich of different species. Most of these species can also grow in "better" places if they get the chance, and their advantage is a high level of adaptability. In a similar manner the crucifers might constitute an environment which requires special adaptation. They are a group of plants that are known to produce a number of secondary metabolites including, 25 different phytoalexins with proven antimicrobial activity (Pedras *et al.* 2000), thiocyanates and nitriles (Taiz & Zeiger, 1991), and proteinase inhibitors (Lorito *et al.* 1994), which might be difficult to metabolise for bacteria that normally compete by rapid growth, while the BCA generalist has adapted to do so.

3. Isolation of and screening for BCAs

3.1 Pre-isolation factors

In classical microbiology, there are relatively few well-known microorganisms that are pathogenic to humans or animals, food and feed spoiling organisms, or organisms that are known as contaminants in sensitive systems (e.g. in the brewery industry or in medical equipments). Most of these organisms have been known for a long time and the knowledge of their metabolism, spectra of sensitivity to antibiotics, and general biology is profound. There are developed methods to separate them from mixed bacterial populations in samples by means of enrichment procedures and/or selective media. In our area of research, this is seldom the option, but with increasing knowledge of the biology of BCAs the methods to select for them are being developed and refined. The approach of enrichment for certain microbial groups has sometimes been used. That enrichment for isolates with high production of lytic enzymes could increase the probability to find disease-suppressive isolates was shown by Berg *et al*. (2002). They cultivated mixed bacterial populations sampled from the rhizosphere in a liquid substrate containing cellulose, chitin, xylan, or casein for five days. Afterwards they isolated bacteria on solid plates, as with the control group, and found that the enrichment procedure had resulted in more than twice as many (7.5% vs. 3.0%) antagonistic isolates. An *in vivo* enrichment approach was used by Landa *et al.* (2002). They grew peas in field soils and when the plants were harvested after 4 weeks the same soil was used for sowing new peas. These were grown for 4 weeks, and so on, for eight cycles. Each time samples of roots were taken and the population of 2, 4-diacetyl phloroglucinol (DAPG)-producing pseudomonads was estimated. Even though this was really not the point with their work, they found that there was a considerable increase of DAPG-producing bacteria from log 4.0 and 4.6 at the first harvest to log 5.0 and 5.5 at the last, in the two tested field soils. But strangely this enrichment was dependent on the pea cultivar, that is, in a parallel experiment with another cultivar the DAPG-producing population was stable at approximately log 5.5 throughout the experiment. Heat-treatment is a well known pre-treatment method that select for spore forming bacteria (Kim *et al.* 1997).

The IODS were probably found as a consequence of some kind of enrichment process (**II**). The plants were sampled from 22 geographically distinct locations and in each one of the 50 samples a pseudomonad of the IODS-type with strong antifungal effect was isolated. This type dominated all the samples but typically only one such colony/sample was isolated. The low variation, i.e. the enrichment for this type of bacteria, I believe, was that the plant samples apparently were subjected to a fermentation (a strong smell resembling H_2S was released when the sealed bags containing the samples were opened). A new isolation was designed to test this hypothesis using Swedish samples. Even though all samples were fermented until the appearance of the characteristic smell, the IODS-type was not found in those samples. Several factors differed from the previous isolation occasion such as; the time of the year, the plant species composition and the sampling country. Hence, the initial microflora prior to fermentation was probably quite distinct from the one in the Swiss samples. Yet another attempt to repeat the enrichment for IODS was made. Samples from Switzerland were obtained from partially the same locations (31 plant samples from 21 locations) and at the same time of the year. However, the IODS-type of bacteria could not be isolated from any of the samples. The whole process of sampling and delivery of samples was out of our control, since samples were sent by mail from Switzerland, and hence there could be many unknown factors that varied between the two isolation occasions, for instance the storage time and temperature. Upon arrival in Sweden, two differences between the two "Swiss" groups were observed: first, the fermentation was more advanced and, secondly, there were more plants and less soil in the samples from the first group than in those used for the second isolation.

Bacteria inhabit all possible niches in the environment, yet, mainly cultivated plants have been used as sources for isolation of potential biocontrol agents. There a dearth of literature records that discusses the selection of bacteria during the isolation process. The origin and type of plant material, as discussed in the previous chapter, can probably have as much influence on the resulting isolate-collection as have the methods used for isolation. The time of the year when plants are sampled can also have an impact on the isolation results (Table 2; Smalla *et al.* 2001). In a study by Johansen *et al.* (2002) the possibly negative effect of a BCA (*P. fluorescens*, CHA0) on barley indigenous microflora was tested *in vitro*. They found a remarkable difference in sensitivity between bacteria isolated from samples collected in May (95% and 62% sensitive *Cytophaga*-like bacteria and fluorescent pseudomonads, respectively) and August (44% and 0% sensitive isolates). In Table 2, the results of ANOVA tests are presented as well as proportion of isolates responsible for disease-suppression equal to, or more than 80% in the greenhouse bioassay, for the different factors analysed. The two last columns show the corresponding results when a group of isolates originating from Switzerland were excluded (labelled Group 4 in **II**). These isolates, which include the IODS, are thought to have great impact on the results and for instance the seemingly good result for isolates first isolated on KB does not remain when the IODS were excluded. This table was done at an earlier time point than the one presented in **II** (before the second "Swiss-isolation"), and with a slightly different grouping and analysis of the results. A factor that was not included in the other analysis is the seasonal difference. In Table 2 (next page) it is clear that isolates

from samples that were collected in the spring have a higher proportion of disease-suppressiveness than those originating from samples collected in other seasons. This was true also when the Swiss isolates were excluded (Table 2). I am aware of that the factors not are independent, for instance the differences observed for different latitudes in Sweden are probably not dependent on the sampling locations but reflects differences in other conditions such as plant species composition and media used for isolation. I don't claim that the figures that are presented in this table

Table 2: Effect of isolation-factors on the frequency of disease-suppressive isolates

Isolation-factor[a] Groups	Total No. of isolates in the group	Proportion of isolates with ≥ 80% disease-suppression (% of total)[b]	P-value (one-way ANOVA) for factors of the same category with / without Swiss isolates	Swiss isolates excluded	
				Proportion of isolates with ≥ 80% disease-suppression (% of total) [b]	Total No. of isolates in the group
Plant:					
Grasses	304	9.9		6.0	268
Brassicaceae	34	29.4		11.5	26
Others	83	7.2	0.001 / 0.008	2.6	77
Media:					
KB	247	13.0		3.6	197
TSA	119	4.2		4.2	119
SEA	55	16.4	0.000 / 0.000	16.4	55
Pre-treatment:					
freezed	150	19.3		4.0	100
not freezed	271	6.3	0.000 / 0.753	4.8	271
fermented	243	12.8		3.6	194
not fermented	178	8.4	0.034 / 0.337	7.9	177
Season:					
autumn	83	1.2		1.2	83
winter	260	6.2		6.2	260
spring	78	37.2	0.000 / 0.000	14.3	28
Latitude:[c]					
47	50	50.0		-	-
56	206	3.4		-	-
60	112	11.6		-	-
68	53	1.9	0.000	-	-

a. Effects of pre-isolation factors on the frequency of disease-suppressive bacteria that was obtained from the group of isolates that was subjected to the same conditions (see also **II**). The isolates from different sets of host plants, isolates obtained from different media (KB=King's medium B, TSA=Tryptic Soy Agar, SEA=Soil Extract Agar), pre-treatment methods previous isolation, and isolates from samples collected at different seasons were compared.

b. The suppressiveness of isolates to wheat seedling blight was evaluated from greenhouse biotest data and expressed as disease-suppressive-index (DSI) on a scale from 0 to 12, where 0 reflected no disease-suppression (all seedlings dead) and 12 full disease-suppression (all seedlings healthy). Bacterial isolates that obtained a DSI of ≥ 9.6 had at least 80 % disease-suppression. Statistical analysis was performed using one-way ANOVA.

c. Latitude 47 denotes isolates with Swiss origin, and the rest are Swedish.

should represent a general truth, rather they are presented as an illustration of the relative importance of selection prior to plating and isolation (plant species, pre-treatments and seasons and geographical locations of sampling) as discussed above, compared to the selection which occurs due to plating on different media

Only the imagination can set the limit as to what other pre-isolation factors that could be interesting to test. For instance, the plants could be inoculated with high levels of pathogen inoculum, stored for some time, and then bacteria that survive this tough competition can be isolated. The low-temperature effect could be worth exploring further (see below), as well as different types of fermentation. Different amendments such as; tryptophane to give IAA-producers an advantage, glycine and iron to select for HCN producers (Defago *et al.* 1990, Blumer & Haas, 2000), or treatment of samples with for instance ozone or H_2O_2 to select for bacteria that are able to degrade ozone and other reactive oxygen species which certainly would give them a competitive advantage in the rhizosphere (Glick *et al.* 1999). Douglas & Deacon (1994) used water stress for selection of fungal BCAs against *F. culmorum*.

3.2 Methods for isolation of rhizobacteria and selection for specific traits

Under this subtitle one can identify two different levels of 'isolation', firstly – all bacteria that are growing on the 'master-plate' are isolated from the plant-roots; secondly – each colony, which is isolated to a pure-culture plate, is isolated from the master-plate. In both these steps there will be a selection of certain types of bacteria. In the first step the conditions used for the isolation will determine which bacteria that will, or will not grow or have a competitive advantage over others. In the second selection it is more of a choice which bacterial colonies we decide to save.

In the first step there are many factors that can be varied; the composition of the media, temperature, exposure to UV-light, pH, addition of certain antibiotics, etc. But in this step it will also be different results depending on the method used for inoculation of the master-plates. After rinsing/washing of the samples, a common method is to put pieces of e.g. roots in a buffer or the like and the do a mechanical separation of bacteria with aid of for instance a stomacher, vortex or sonicator (Kempf & Wolf, 1989; Milus & Rothrock, 1997; Raaijmakers *et al.* 1997; Neiendam Nielsen *et al.* 1998; Pierson *et al.* 1998; Mc Spadden Gardener *et al.* 2000; Kahn *et al.* 2001; Kaiser *et al.* 2001; Landa *et al*. 2002; Nielsen *et al.* 2002). The suspensions obtained are often far to concentrated to be plated directly so there is often a need to make a dilution series in order to obtain single colonies. Another option is to use surface sterilisation methods previous plating, which depending on the time and substance used for sterilisation, will make dilution necessary or not. When the aim is to isolate endophytic bacteria this would of course be the best option (McInroy & Kloepper, 1995), but also bacteria that are residing in the inner part of the cortex could be isolated with such methods. At our unit we have used a plate inoculation method where roots were used directly for

isolation, without dilution and without surface sterilisation (Alström, 1987; Åström, 1991; Hökeberg, 1998, **II**).

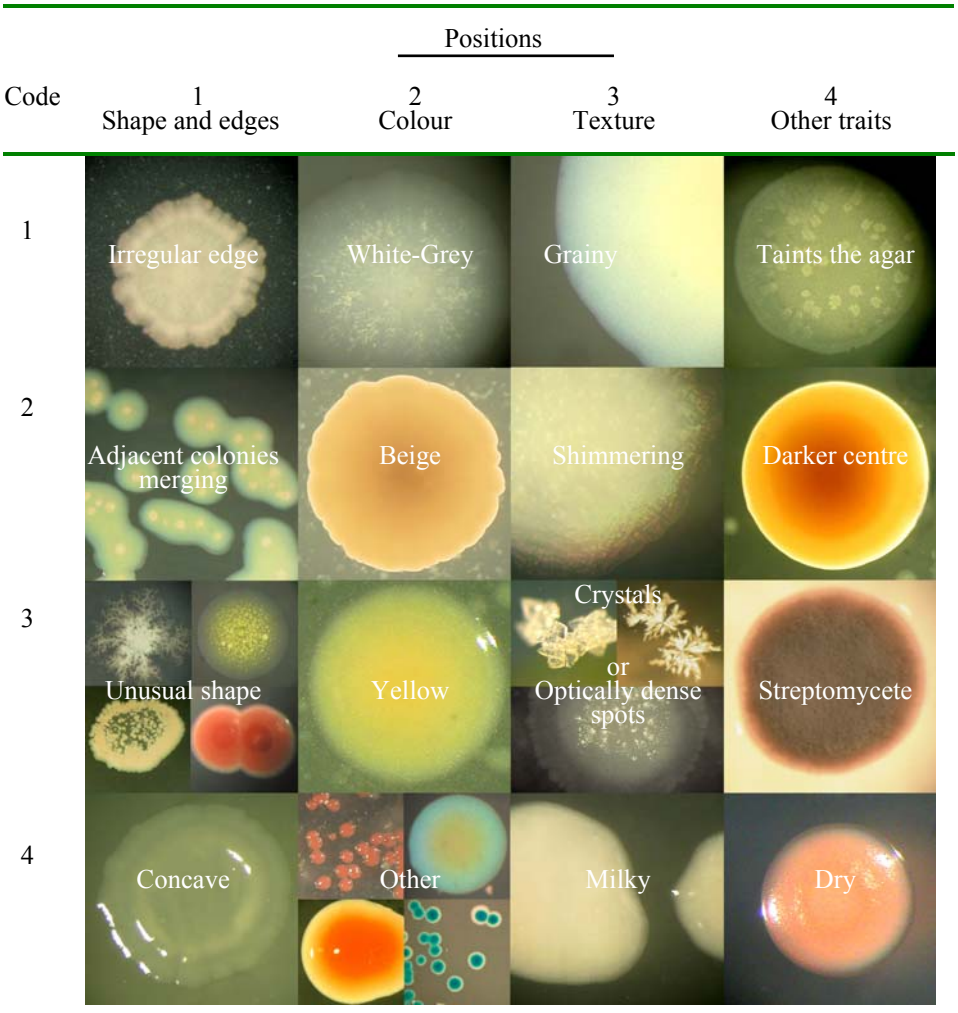
In this work, only psychrotrophic (see Glossary) bacteria were studied, since all of them were initially isolated after growth at +1.5°± 1.0° C. One of the target diseases was snow mould, which develops near 0°C, and hence it was logical to search for bacteria that were able to grow at these low temperatures (**I** & **II**). A surprising finding was that some of the psychrotrophic isolates were also able to grow and be disease-suppressive at much higher temperatures. Strain MF 30 (an atypical *P. veronii*, see Table 5 in **II**) suppressed development of fusarium wilt in tomato that was cultivated in greenhouse at temperatures around 25-30°C (**IV**). There are only a few reports where the influence of the temperature has been emphasized. The BCA, *Bacillus sp*. (L324-92), had suppressive effect on several soilborne diseases and the authors believed that the ability of strain L324-92 to grow at 4°C probably contributed to the positive effect (Kim *et al.* 1997b). This strain was selected in growth-chamber experiments carried out at 15°C (Kim *et al.* 1997a). The PGPR-strain *P. putida* GR12-2 can resist low temperatures by producing an antifreeze protein and promotes plant root elongation at 5°C (Sun *et al.* 1995). This strain was part of a collection of nitrogen-fixing (diazotrophic) pseudomonads with ability to colonize roots of canola (*Brassica campestris*) at low temperatures. The diazotrophic strains were enriched for and selected in order to use them as bio-fertilizers that would work at low temperatures (Lifshitz *et al*. 1986).

The second isolation step can imply selection of bacteria with specific characters of those that are growing on the (mostly) mixed culture master plates. If selective media have been used and the aim was to only isolate for instance; DAPG-producers, they reveal themselves by producing a brown pigment that diffuses to the agar (Raaijmakers *et al.* 1997); protease producers causes a clearing zone in skim-milk agar (O'Sullivan *et al.* 1991; Borowicz, 1998), chitinase producers causes a clearing zone in agar containing colloidal chitin (Frändberg, 1997); siderophore producers colours the agar bright yellow when grown on Kings' medium A and B and has fluorescence in UV-light (Stolp & Gadkari, 1981, Borowicz & Saad Omer, 2000), *Cytophaga*-like bacteria have a particular orange colony morphology and turn red when treated with 10% KOH (Johansen *et al.* 2002). A random selection of a certain number of isolates has been used (Milus & Rothrock, 1997; Kaiser *et al.* 2001). Another approach is to use molecular markers that hybridises with colonies that have the desired trait. Giacomodonato *et al.* (2001) used a primer specific for conserved regions in genes coding for peptide synthetases, which often are involved in the synthesis of different antibiotic substances, as a marker for isolation of potential BCAs from suppressive soils. There are also primers selective for DAPG- and phenazine-producing strains and this has been used to select for such strains and characterise their geographical distribution (Raaijmarkers *et al.* 1997).

A common way to select isolates is to pick all colonies that are morphologically different (McInroy & Kloepper, 1995; Nautiyal, 1997; Berg *et al.* 2002). That approach was also used in this work, and it is amazing to see how many different shapes and colours bacteria express when forming colonies. During the isolation I

tried to categorise these and the result is presented in paper **II**. First I used a code with six categories and two to ten characters in each category, but it was too many different characters and in the analysis it was difficult to draw conclusions since the groups were not at all equally big. Then the 4x4 matrix was constructed, giving a four digit code to each isolate, and this is better but it could still be improved. Each isolate was observed with a stereomicroscope and the characters were coded using the matrix in Figure 2 in **II**. As an example; a colony looking like the one illustrating "Darker centre" in Figure 3, would obtain a code 0412. In the first position none of the mentioned characters fitted, hence the zero. Below is a figure that gives an idea of the characters that was searched for and how they look like (Figure 3).

Figure 3 Illustration of the characters that were used to code the colony morphology of rhizobacteria



3.3 The concept of field-correlation

Several authors remark that screening systems that resemble field conditions are more likely to result in the selection of effective biocontrol isolates. (Dowling & O'Gara, 1994; Knudsen *et al.* 1997; Dickie & Bell, 1995; Glick, 1999). But within the term "field conditions" there is a quite complex mixture of factors that have more or less impact on how well a specific bacterial isolate will adapt to these conditions. A factor that often has been mentioned is to use a low temperature during screening, and even though it has not often been practiced, in the cases were it were have shown promising results (Hökeberg *et al.* 1997, Kim *et al.* 1997a & b, **I**). In the present work, isolation, bioassay-development, screenings and field-experiments, were performed in a parallel manner. The first summer a representative selection of the isolates available at that time were tested in a field-screening. The results obtained were then used to evaluate the relevance of the bio-assay until then used. Adjustments of the bio-assay were done and the results affected also the parameters used for further isolation, for instance the different media used in the beginning were abandoned and only Kings' medium B was used in further isolation. The next spring new field-experiments were performed and new adjustments done. The resulting bio-assay and the field results for included isolates correlates well (r-sq = 0.72), (**I**). The conclusions of the above are that an early involvement of field experiments is crucial for the outcome of the screening work. It is also better to abandon methods that not do reflect what is happening under field conditions even though it can make the results less comparable.

Another aspect that has been extensively discussed in the literature is the colonisation ability of the bacteria, the so called, "rhizosphere competence". There are different opinions about how crucial to biocontrol efficacy this ability is. Depending on which mechanism(s) that are involved in the disease-suppression the extent of the colonisation, both spatially, temporally and quantitatively, would be more or less critical. The term "rhizosphere competence" is, in my opinion a bit confusing and vague. I think it would be more informative to talk about ability to colonise and specify to what extent. Even though bacteria that are introduced to the rhizosphere have an advantage of the relatively big population (Bull *et al.* 1991; Jjemba & Alexander, 1999), the introduced bacteria both influence and are influenced by the other microorganisms that are already present (Fukui *et al.* 1994 a & b; Jeong *et al.* 1997; Pierson *et al.* 1998; Rainey, 1999; Stepensen Lübeck *et al.* 2000). This fact and the often observed inconsistency in performance of BCAs has inspired many authors to try to introduce mixtures of BCAs (Pierson & Weller, 1994; Duffy & Weller, 1995; Duffy *et al.* 1996; Janisiewicz, 1996; Leeman *et al.* 1996; Vavrac *et al.* 1996; Schisler *et al.* 1997; Mao *et al.* 1998). To find a compatible pair of microorganisms can be quite difficult, at least if the mix consists of two bacterial strains. This was experienced by Schisler *et al.* (1997) who tested 18 strains in 90 different combinations for effects against fusarium dry-rot of potatoes. This quite ambitious work yielded 16 pairs that showed synergistic effects, that is, the combination had better control of the dry-rot than each strain individually. Pierson & Weller (1994) tested combinations of more than two isolates in field experiments and found some compatible mixtures, which had a yield increasing effect on wheat. An interesting observation in their work was that

some of the strains that were components of successful mixtures had strong antagonistic effects on many of the other strains in *in vitro* tests. The authors speculate that the intercompetition among the strains in the mixtures in fact makes them better BCAs.

A more common approach has been to combine bacteria and fungi and various fungal species have been tested; *Trichoderma* spp. (Duffy *et al.* 1996; Vavrac *et al.* 1996); *Gliocladium virens* (Jeong *et al.* 1997; Mao *et al.* 1998); *G. graminis* var. *graminis* (Duffy & Weller, 1995); and *Acremonium rutilum, F. oxysporum*, and *Verticillium lecanii* (Leeman *et al.* 1996). Often the combinations are compatible and the major gain is by using combinations of bacteria and fungi the same effect is obtained but with lower populations of the BCAs (Leeman *et al.* 1996; Jeong *et al.* 1997), which in part justify the hypothesis by Pierson and Weller, mentioned above. In a study by Woo *et al.* (2002) cell wall degrading enzymes prepared from a *Trichoderma* sp. had a positive effect on the biocontrol potential of a bacterial BCA (*P. syringae*), while lipodepsipeptides prepared from the bacterium had less effect when combined with the living BCA fungus. Hence a possible trait of successful BCAs could be the compatibility with *Trichoderma* spp. in the rhizosphere. The research-area of combining microorganisms (community approach) to obtain synergistic effects has been much studied in the field of post harvest diseases, where yeasts and lactic acid producing bacteria are two major organism-groups studied (Janisiewicz, 1996; Frändberg, 1997; Petersson, 1998).

4. Mechanisms of disease-suppression

This is a subject that has been discussed in many more or less complete review articles and books, written by experienced authors thus I recommend the interested reader to consult some of the references given below. Examples of books, chapters in books, and review articles treating this subject are: (Baker & Cook, 1974; Mukerji & Garg, 1988; Campbell, 1989; Defago & Haas, 1990; Defago *et al*. 1990; Thomashow & Weller, 1990; Dowling & O'Gara, 1994; Dunne *et al.* 1996; Thomashow & Weller, 1996; Boland & Kuykendall, 1998; Glick *et al*. 1999; Whipps, 2001; Gerhardson & Wright, 2002).

Below is an overview of different modes of action – or mechanisms, which normally are involved in the phenomenon we call disease-suppression. To make an efficient BCA there are, in most cases, several of these mechanisms that are considered to be necessary, and there are no clear-cut borders among the different mechanisms (Duijff *et al*. 1998; Bull *et al.* 1991; Chin-A-Woeng *et al.* 2001; Nielsen *et al.* 2002). In some cases it has been shown that one bacterial strain can be disease-suppressive by using different mechanisms in different host plants. Maurhofer *et al.* (1994) observed that a mutant of the BCA *P. fluorescens* CHAO, deficient in the production of pyoluteorin was able to suppress disease caused by *Pythium* sp. in cucumber but not in cress. The BCA *P. fluorescens* WCS417 has been shown to have a broad spectrum of disease-suppressive ability in various cropping-systems, and different mechanisms have been suggested to be responsible for the effects (Leeman *et al*. 1995). There are also indications that the *P. veronii*, strain MF 30 works with different mechanisms when suppressing wheat seedling blight caused by *F. culmorum*, and when the target disease is wilt of tomato, caused by *F. oxysporum* f.sp. *radicis lycopersici*. (**I**, **II**, **IV** & Konnova *et al*. 1999)

4.1 Antibiosis – production of antimicrobial metabolites

Antibiosis is the most studied and maybe also the most common way by which introduced pseudomonads are able to suppress disease development caused by fungal pathogens (Ligon *et al.* 2000). This could be a consequence of the common method to use dual cultures (Figure 4) when screening for BCAs in the early years of biocontrol research.

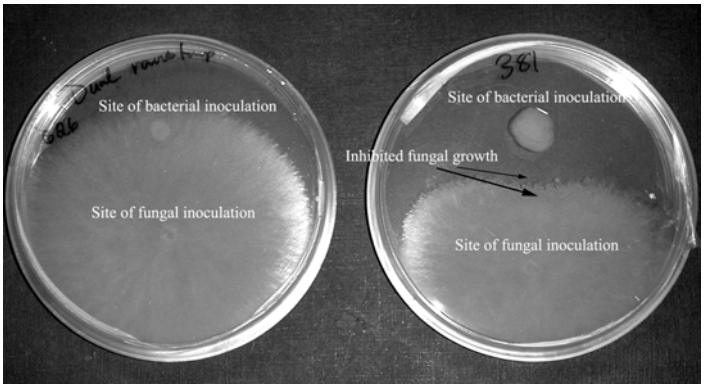


Figure 4

Dual cultures with the fungus F. culmorum and a bacterial strain that not has any in vitro antibiosis (left) and one strain that has (right)

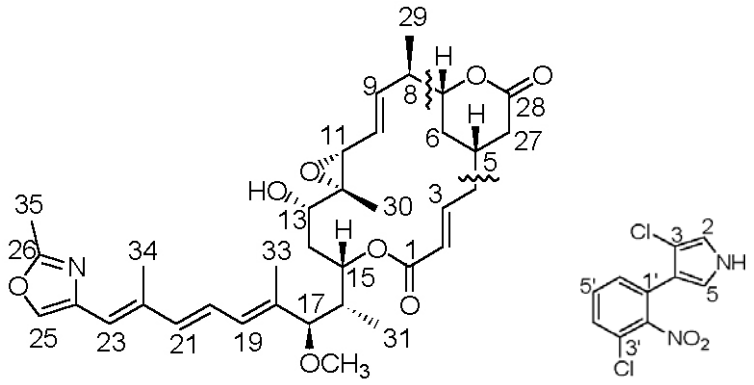
The number of antifungal substances produced by florescent pseudomonads is high, even though convincing proof of activity in disease-suppression *in vivo* not is available for all of them. The secondary metabolites produced by this group were compiled by Leisinger & Margraff (1979) and consisted of around 75 substances. Dowling & O'Gara listed 70 compounds (based on the Leisinger & Margraff review), and mentioned eleven as implicated in biocontrol of plant diseases. In a recent paper by Ligon *et al.* (2000), nine substances are mentioned, eight of those in the Dowling & O'Gara review and 2,3-deepoxy-2,3-didehydro-rhizoxin (DDR), which was identified in one of the IODS (Hökeberg, 1998; Svensson, 1999; **II**). The ten metabolites that are involved in biocontrol that were listed in the references above are:

- Pyrrolnitrin
- Phenazines (Pyocyanin)
- 2,4-diacetyl phloroglucinol (DAPG)
- c-Acetyl phloroglucinol
- 2,3-deepoxy-2,3-didehydro-rhizoxin (DDR)
- Hydrogen cyanide (HCN)
- Pseudobactin B10
- Agrocin 84
- Pyoluteorin
- Ammonium

Some of the metabolites mentioned in these papers were also found to be produced by a number of the strains used in this work, and in paper **III** a contribution to the list of substances produced by *Pseudomonas* spp. with a previously undescribed substance is presented. The new substance is a triene named pseudotrienic acid A, which has antibacterial effect *in vitro* against *P. syringae* pv. *syringae* and *Staphylococcus aureus* (**III**). The new substance was isolated from one of the IODS together with two metabolites that are known to be involved in biocontrol in other strains; DDR (Wright *et al.* 2003) and pyrrolnitrin (Pfender *et al.* 1993).

Some metabolites seem to be very commonly produced in nature, and it seems to be relatively easy to isolate bacteria that are able to produce them. DAPG-producing strains have been found worldwide; in Europe, (Keel *et al.* 1990; Fenton *et al.* 1992; Shahanan *et al.* 1992; Georgakopoulos *et al.* 1994; Neiendam Nielsen *et al.* 1998; Berg *et al.* 2000; Waechter Alsanius *et al.* 2002), in Asia (Yuan *et al.* 1998; Kamei & Isnansetyo, 2003), and in America (Vincent *et al.* 1991; Pierson & Weller, 1994; Raaijmakers & Weller, 2001; Landa *et al.* 2002), in habitats as different as on a red alga in the sea (Kamei & Isnansetyo, 2003) and in disease-suppressive soils (Mc Spadden Gardener *et al.* 2000). Also pyrrolnitrin (Pfender *et al.* 1993; Hill *et al.* 1994; this work **III**) and phenazine-producing strains seem to be quite common (Kanner *et al.* 1978; Thomashow & Weller, 1988 Levy *et al.* 1989; Brisbane *et al.* 1990; Bull *et al.* 1991; Georgakopoulos *et al.* 1994; Pierson III *et al.* 1994; Mazzola *et al.* 1995; Slininger *et al.* 1996; Chin-A-Woeng *et al.* 1998). Others are not as easy to find and isolate as discussed in paper **II**, even though they might be commonly occurring.

Figure 5 Structure of two of the three antibiotics found in strain MF381-IODS



DDR *Pyrrolnitrin*

A critical point of view is that once a substance has been described as antifungal, it is much easier to find it again in other strains. Now when many of the genes that are involved in the synthesis of these substances are characterized, it is easier still to identify strains that produce them. By using probes specific for the DAPG-producing strains they have been further grouped and characterised. For instance do they have different abilities to colonise pea roots (Landa *et al*. 2002), or to suppress the take-all disease (Mc Spadden Gardener *et al.* 2000). These primers were also used by Berg *et al.* (2002), and they found a high frequency of isolates originating from strawberry (63%) and potato (74%) rhizospheres that harbored the *phlD*-gene. As mentioned above some of these "common" metabolites were found among the isolates in this work, but those findings were rather accidental, and there was no systematic search for them. Nevertheless I suppose that several of the other isolates with high DSI-values (**I** &**II**), are most likely able to produce different siderophores and/or HCN – siderophores because they have fluorescence when grown on KB (Table 5 in **II**), and HCN because I believe that this compound has a vital role in the cell metabolism of Gram-negative bacteria (see below).

The mechanisms of the individual substances can be fungistatic or fungitoxic, or both, depending on the dose. The mechanism of DAPG is probably membrane disruption, since the sensitive organism lyses after a short exposure, Gram-positive bacteria being more sensitive than Gram-negative ones (Kamei & Isnansetyo, 2003). The production of HCN is a common and well known mechanism used by BCAs (Ellis *et al.* 2000). Likewise, many plants produce this substance for protection. HCN (or in fact CN^-) blocks cellular respiration by binding to the iron-containing heme-group of cytocrome oxidases and other carriers of oxygen, and the toxic effect increases at low oxygen pressure (Negherbon, 1959; Taiz & Zeiger, 1991). The mechanism of DDR is fungi static, since the substance binds to β-tubulin and inhibits their assembly, hence making cell division impossible

(White *et al.* 2002). The molecule is extremely active and effects are observed already at pikomole-levels (Aoki *et al.* 2003). All the above mentioned metabolites seem to be able to suppress fungi in an unspecific manner (they have been reported as the active substance in many different systems, see references above), thus they can be assumed to be quite toxic. It can be questioned what the real environmental benefit is of using them. Spreading large quantities of such organism can make the long-term risk assessment difficult (de Leij *et al.* 1995; Hokkanen & Lynch. 1995; Stephensen Lübeck *et al.* 2000; Schmalenberger & Tebbe, 2002). This is a point in concern by the authorities and can make the registration procedure both difficult and expensive, when a BCA is developed into a biopesticide. Since the focus tends to be on the metabolite(s) rather than the whole organisms, the BCAs are treated like any other active substance used for control of plant pathogens. There are some obvious benefits though, using these *'in situ* chemical factories'. The amount of active substance needed for effect is a small fraction of that of chemical pesticides, and since the natural antifungal or fungistatic compounds are produced in nature it is more likely that their turnover rate is shorter than for the synthetic substances. It has been shown that 'biocontrol' actually can be a natural process. In fields with monoculture of wheat the phenomenon referred to as take-all decline has been explained by an increase of 2,4-diacetyl phloroglucinol producing bacteria, hence a natural selection for this type of bacteria is occurring (even though monoculture *per se* is rather unnatural) (Mc Spadden Gardener *et al.* 2000). Another example of a similar natural process is the phenomenon of suppressive soils (Persson, 1998; Giacomodonato *et al.* 2001).

4.2 Triggering of host defence mechanisms

The plant has various strategies to defend itself, and if it is attacked there are a number of reactions that are initiated. The biotrophic pathogens which have a history of co evolution with their respective host have developed strategies to avoid "pushing the red alert button". That is, to avoid triggering the plants' inducible defence system. Once this system is alerted there are few obligate parasites that manage to infect such a plant. However, the other type of pathogens – the necrotrophs, who not are dependent on living tissue, can in fact be more destructive on a plant that is alerted, since they are not restricted by, for instance, a hypersensitivity reaction, on the contrary they benefit from it (Govrin & Levine, 2000). The previous conclusion was based on observations with *Arabidopsis thaliana* and *Botrytis cinerea*. In another work this conclusion is contradicted by observations made by Ongena *et al.* (2002), who shoved that a *P. putida* (strain BTP1) was able to induce resistance in bean against *B. cinerea*. The explanation to the contradictory results obtained by these two groups can be that they observed two different types of induced resistance, but also other possible reasons can be at hand.

The two types of induced resistance that have been characterised are: systemic acquired resistance (SAR) and induced systemic resistance (ISR). The SAR-type is the most studied since it is normally related with pathogenicity, and has connections with race specificity and the hypersensitivity response in "non-hosts",

which is an effective defence reaction against biotrophic pathogens like rusts and downey mildew. The SAR-type of resistance also involves salicylic acid and accumulation of pathogenesis-related proteins (PR-proteins) and phytoalexins. The PR-proteins are plant produced and accumulates rapidly in a plant expressing SAR, and has been characterised owing to this trait, rather than because of their antimicrobial activity. These proteins are of different types and grouped in "families". Commonly they are chitinases, β-1,3-glucanases, proteinase inhibitors and peroxidase (van Loon, 1997). The SAR has also been connected with stress and can be induced by certain chemical compounds (Kuhn, 1987, Kuć, 1987, Hammerschmidt *et al*. 2001). The ISR-type, on the other hand, does not cause the hypersensitivity response and usually there is not accumulation of PR-proteins and phytoalexins as with the SAR-type. Typically the ISR-type of response is induced by lipopolysaccarides (LPS), (Leeman *et al*. 1995), siderophores or salicylic acid produced by bacteria (van Loon, 1997; van Loon *et al.* 1998). A new not yet characterised compound named "Cx", which is produced by *P. putida* (strain BTP1) has also been shown to initiate ISR (Ongena *et al*. 2002). In summary:

- SAR is induced by pathogens, stress, and some chemicals. It leads to hypersensitivity reaction and accumulation of PR-proteins. Salicylic acid is a signal involved.
- ISR is induced by PGPRs, by means of plant recognition of LPS, siderophores, bacterial produced salicylic acid. No hypersensitivity reaction and no accumulation of PR-proteins.

But this is a role with modifications, and it is not as clear-cut borders between the two systems as was once believed (Hammerschmidt *et al*. 2001), nor is SAR stronger or more persistent than ISR. Hoffland *et al.* (1996) showed that ISR induced by the PGPR pseudomonad, (strain WCS417) was even stronger than the resistance obtained with an inducer (*P. syringae* pv. *tomato*) causing SAR, including the hypersensitivity reaction and accumulation of three different PR-proteins. The PGPR triggered the defence system of radish in a manner that made it resistant to a broader spectrum of pathogens than *P. syringae* did. Duijff *et al*. (1998) also compared strain WC417 with an inducer of SAR (a non-pathogenic *F. oxysporum,* Fo47), but in tomato. It was again demonstrated that the ISR was stronger than the SAR, but the biocontrol effect was stronger with Fo47 than with WCS417. Their report demonstrated the importance of securing that the inducer and the pathogen are properly separated, in order to be able to draw conclusions. This is also the reason why it was not concluded in **IV** that ISR was the mechanism by which, strain MF 30 (atypical *P. veronii*) suppressed fusarium wilt of tomato. Later work however, has confirmed the ISR-action of MF 30, and purified LPS of this strain were able to cause this response in tomato (Konnova *et al*. 1999).

In biocontrol, two different strategies of inducing plant resistance have been deployed; one is to "vaccinate" the plant with a non-pathogenic, close relative to the pathogen (Duijff *et al*. 1998) or killed or separated extracts of the pathogen (Kuć, 1987); the other is to screen for rhizobacteria that have the ability to induce plant resistance (van Loon *et al.* 1998). Crops that often are connected with this type of biocontrol are; tomato (*Lycopersicon esculentum*), (Fuchs *et al*. 1997;

Duijff *et al*. 1998; Konnova *et al*. 1999; Jetiyanon & Kloepper, 2002; **IV**), cucumber (*Cucumis sativus*), (Wei *et al.* 1996; Jetiyanon & Kloepper, 2002), radish (*Raphanus sativus*), (Leeman *et al*. 1995; Hoffland *et al*. 1996), and bean (*Phaseolus vulgaris*), (Ongena *et al*. 2002). There are not so many reports concerning induced resistance in cereals, but in a review by Ramamoorthy *et al.* (2001) some examples of ISR in rice (Oryzae sativa) are mentioned. In the summary of the papers presented at the First International Symposium on Induced Resistance to Plant Diseases (2000) there are examples of induced resistance in barley (*Hordeum sativus*) and rice (Hammerschmidt *et al*. 2001). In work by Mohammadi & Kazemi (2002), it is demonstrated that also in wheat there are possibilities of biocontrol by means of induced resistance. They were able to induce resistance against *F. graminearum* in a susceptible cultivar by spraying the heads with heat-killed mycelial wall preparations. They also attempted treatment with salicylic acid, but this had no effect.

SAR/ISR is a mode of action in biocontrol that has many advantages – it often operates on a broad spectrum of pathogens, fungi, bacteria, viruses and sometimes also on pests such as nematodes and beetles (Hoffland *et al*. 1996; Ramamoorthy *et al*. 2001; Hammerschmidt *et al*. 2001; Jetiyanon & Kloepper, 2002). The induced resistance can also have a relatively durable effect, but this varies with both the crop and the inducing agent (Wei *et al.* 1996; Ramamoorthy *et al*. 2001). PGPR strains applied to seeds induced systemic resistance and resulted in yield increases of cucumber under field conditions. The tests were performed two consecutive years and the results were repeatable (Wei *et al.* 1996).

4.3 Competition for nutrition and space – colonisation traits

There are examples where mutants of biocontrol strains that are deficient in the production of antimicrobial substances, are almost as efficient in biocontrol as the wild type (Kempf & Wolf, 1989; Kraus & Loper, 1992; Maurhofer *et al.* 1994). Those examples call attention to the importance of colonisation ability and other competitive traits of BCAs.

4.3.1The process of colonisation

The pseudomonads are motile and swim towards an energy source, which they can sense as a gradient through receptors on their cell surfaces, a process called **chemotaxis** (van Bastelaere *et al.* 1999). Once a nutrition source is encountered, the cells get **attached** to the surface, a process that is quite fast and irreversible (James *et al.* 1985; Glandorf *et al.* 1994; Hood *et al.* 1998). Some described components that are involved in this process, called **adhesion**, are bacterial pili, a special root-adhesive protein and a plant-produced glycoprotein complex termed agglutinin (Glandorf *et al.* 1994; Glick *et al.* 1999). Adhesion can also be improved by the presence of divalent cations, such as Mg^{2+} and Ca^{2+} (James *et al.* 1985; Hood *et al.* 1998). When the bacterial cell is about to get attached to a surface it starts to move in a particular way that is called twitching motility (Darzins & Russell, 1997).

There is an alternative term for the events described above; **biofilm formation**, which is used in areas dealing with a somewhat more technical use of bacteria, such as bioremediation (O´Toole & Kolter, 1998). When the bacteria once are attached they cannot detach, but this is probably not be true for later generations that are produced in the **micro colony** that is formed. The bacterial colonisation pattern on the root surface is typically uneven, with higher concentration of bacteria in natural cracks and crevices (Fukui *et al.* 1994c; Hood *et al.* 1998). In experiments studying the organisation of bacterial colonies, Shapiro (1998) observed that bacterial cells had a higher priority to maximise their cell-cell contact, rather than being in contact with the nutrition source. When the colony reaches a certain critical density, there is a change in the expression of genes that are regulating for instance the production of secondary metabolites (see below) and formation of sexual pili (Swift *et al.* 1996).This shift in the expression of genes is referred to as **quorum sensing** (see below). The **mucigel** described in chapter 2, which is formed both by the plant and the bacteria, might aid the bacterial spread on the roots. It has been shown that biosurfactants are important and commonly produced by rhizobacteria (Gerard *et al.* 1996; Nielsen *et al.* 2002).

4.3.2 Competition

Any bacteria that are introduced in the rhizosphere will encounter quite rough neighbourhoods, and the ability to use a specific compound as an energy source, that not all microorganisms are able to use, can provide them with a competitive advantage (Lugtenberg *et al.* 2001). The BCA *Enterobacter cloacae*, EcCT-501R3 controls the damping-off disease, caused by *Pythium ultimum* by metabolising linoleic acid. This pathogen survives in soil as resting sporangia, which germinate in the presence of a host. A critical compound for germination is linoleic acid, which breaks the fungistasis and is present in small amounts in root exudates. This was demonstrated by van Dijk & Nelson (2000) by using a mutant deficient in β-oxidation genes (fatty acid catabolism). In work by Moulin *et al.* (1996), a florescent *Pseudomonas* sp. strain CH31 was observed to reduce root colonisation of *Pythium aphanidermatum*, causing root rot in cucumber, but in this case the mechanism has not yet been confirmed. It has conversely been shown that a pathogenic *P. ultimum* has the ability to suppress the expression of certain genes in a biocontrol pseudomonad (*P. fluorescens* F113) hence making it lesser fitted to the rhizosphere environment (Fedi *et al*. 1997). The ability to use organic acids seems to be an important competitive advantage in the rhizosphere, and a monitor of potential biocontrol activity (Goddard *et al.* 2001; Khan *et al.* 2001).

The ability to use and compete for inorganic compounds is another important aspect, which would determine whether a potential BCA will be successful or not in suppressing a pathogen. Iron is one of the resources that can limit growth of plant pathogens and one well-known mechanism of competition in the rhizosphere. Many bacterial isolates used for biocontrol have the ability to produce iron-chelating compounds, siderophores (Schippers *et al.* 1987; Defago *et al*. 1990; Dowling & O'Gara, 1994). In recent time the importance of the mechanism to starve the pathogen of iron has been questioned (Weisbeek & Gerrits, 1999), but nevertheless the ability to produce siderophores gives a competitive advantage in environments where soluble iron is scarce (Weisbeek &

Gerrits, 1999). See also below concerning the regulatory role of iron in gene expression. Bacteria that are able to reduce Manganese can have a positive effect on plant vigour (Elmer, 1995), and Zinc improved biocontrol of fusarium crown and root rot of tomato by *P. fluorescens* CHA0. The production of fusaric acid by the pathogen, which had an inhibitory effect on the production of DAPG and pyoluteorin, was inhibited by amendments with Zn^{2+} (Duffy & Defago, 1997). It was demonstrated by Percheron *et al.* (1995) that Zinc drastically increased exoprotease production of a pathogenic *Burkholderia* strain. .

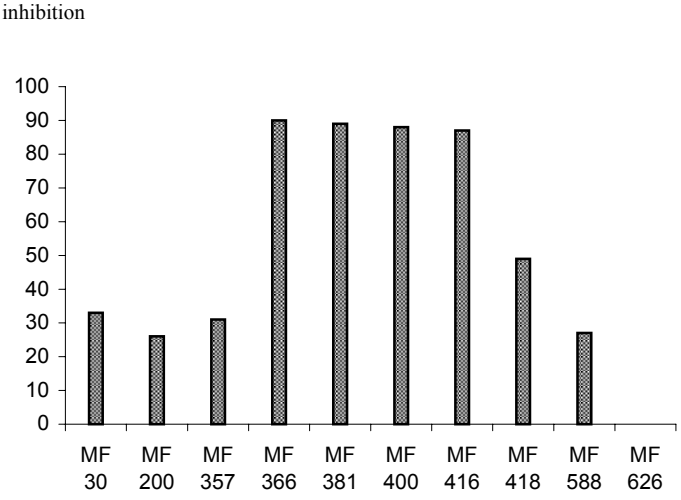
HCN production has often been suggested to be a mechanism of biocontrol (Voisard *et al*, 1989; Defago *et al*. 1990; Pierson & Weller, 1994; Blumer & Haas, 2000), where the role of this substance is to antagonise cyanide sensitive pathogens. Many microorganisms have systems to defend themselves against cyanide, such as cyanide insensitive cytocrome oxidases (Tempest & Neijssel, 1987) or enzymes that can detoxify the cyanide by converting it to formamide (van Etten & Kistler, 1984). To me it is puzzling why bacteria have this production of HCN. It seems to be quite a common trait among rhizosphere bacteria, and in my opinion there has not been any satisfactory explanation as to why they do it. In addition the production of siderophores is a very common and to some extent unexplained feature of rhizobacteria. Could these compounds have another role as well? Somehow there seems to be a connection between iron and HCN, which in turn seems to be connected to the respiration. I have been contemplating this, and a (wild) speculation of how these events could be connected is as follows: when oxygen, but not iron is limiting, the bacteria start producing HCN (Blumer & Haas, 2000), but in doing so they risk damaging their own cytocrome oxidase if the cyanide ions are not immobilised. A candidate mechanism is possibly that $Fe(OH)_3$ reacts with the cyanide ions to form a complex, $[Fe(CN)_6]^{3-}$, (hexacyano-ferrate-III). The mineral $Fe(OH)_3$ is insoluble at neutral pH, but the bacteria are perhaps able to create a local high proton-density and thus making it possible to use this source of iron. The siderophores with their special structure may serve as the site of this reaction, holding the iron ion while the unknown enzyme is attaching the cyanide ions. The bacterial cells might then be able to transport this complex across the membrane while (in lack of oxygen) using it as a temporary electron acceptor reducing the iron (from Fe^{3+} to Fe^{2+}). The $[Fe(CN)_6]^{4-}$ will then probably spontaneously react with water and the hydrogen cyanide will be released. In the same process this will also increase the Fe^{2+} to benefit the needs of the plant. To take this speculation even further, the production of HCN might in fact not have as a main purpose to be armour in the rhizospere competition (Voisard *et al.* 1989), but instead it is a survival strategy that bacteria use to get rid of excess nitrogen. Especially when oxygen is limiting bacteria need to get rid of nitrogen since the levels of NH_3 otherwise can become toxic to the cells. The reduced hexacyano-ferrate-II-complex or the HCN would thus be functioning as a bacterial equivalent to urea in eukaryotes and ammonium in water living organisms. To test this hypothesis would of course be another PhD-project. I made a search for hexacyanoferrat and was surprised to find a reference where this compound actually was used as a terminal electron acceptor in a biological system, or at least a semi biological system (Bottger *et al.* 1995). The report gives evidence of the mechanisms behind NADH-oxidation over a membrane where

maize vacuoles were used to study the membrane transport of electrons. My knowledge of chemistry is unfortunately not as great as I would like it to be, and the speculation above might be totally out in the blue, but it was funny to think in new directions and it made me repeat a lot of 'old' knowledge. This whole mental diversion was probably provoked by half a single sentence in the paper of Blumer & Haas (2000) that said: ' Whilst the role of HCN in bacterial physiology remains obscure ...'.

4.3.3 Parasitism

Sometimes the biocontrol effect is attributed to the production of extracellular lytic enzymes like chitinase; (Chernin *et al.* 1995; Frändberg, 1997; Neiendam Nielsen *et al.* 1998; Berg *et al.* 2001), proteases (O'Sullivan *et al.* 1991; Sacherer *et al.* 1994; Dunne *et al.* 1997; Borowicz, 1998), and/or β-1, 3 glucanase (Friedlender *et al.* 1993; Berg *et al.* 2001). As stated initially – there is no clear-cut border between what should be considered a biocontrol mechanism and, for instance, high competitive ability. As an example, the ability of a bacterial strain to produce chitinase does not automatically imply that it will be used actively against the pathogenic fungus. In fact it has, to my knowledge never been demonstrated that bacteria are using chitinases to attack a living fungus. However, concerning the mode of action of DAPG, which is membrane disruption followed by lysis of exposed cells. Maybe it is a matter of definition, but the mechanism of DAPG producing strains is maybe not much different from what is usually called parasitism. The mechanism is often called mycoparasitism and is a common mechanism used by biocontrol fungi, such as *Trichoderma* sp. and *Gliocladium* sp. (Jeffries, 1994) and *Pythium oligandrum* (Deacon, 1984; Baker, 1987; Davanlou *et al.* 1999). Apart from rapid lysis of the counterpart there are other types of parasitism as well, some parasitic fungi are even obligate parasites (hyperparasites) (Campbell, 1989).

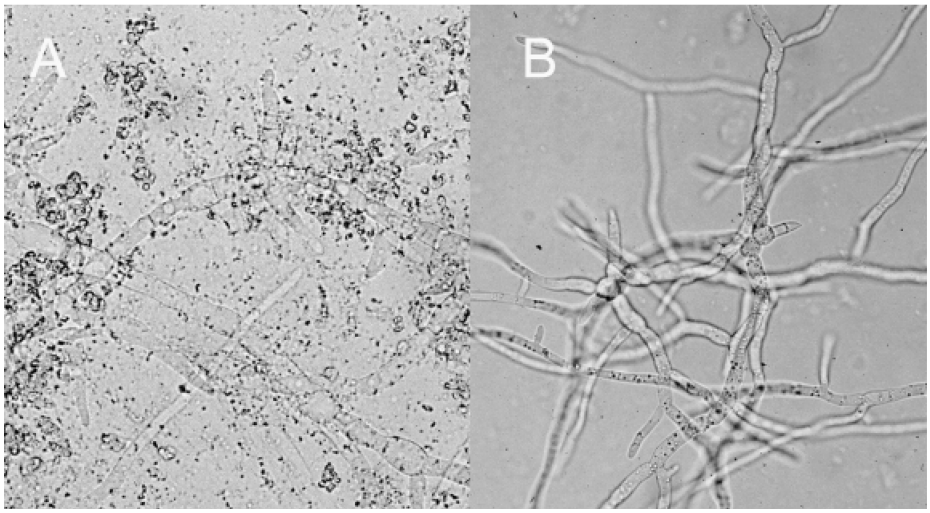
Figure 6 Dual culture inhibition of F. culmorum by rhizobacteria



Some observations concerning strain MF 626 (an atypical *P. agglomerans*); indicate that one biocontrol mechanism of this strain actually is to physically attack the living fungus. In spite of much effort in trying to isolate active

metabolites from this isolate, we failed to do so with the methods used in **III**. The cell-free supernatant of this strain had no *in vitro* inhibitory effect on test fungi and bacteria of nine species (**II**) or on *F. culmorum* in dual culture tests (Figure 6). In spite of this, MF 626 has shown high and repeatable disease-suppression both in field and greenhouse experiments (**I**). However, one result on the chemical end was obtained. When the cell pellets were lysed with acetone and then fractionated by a similar setup as the one used in **III**, an active fraction was identified. The substance that had antifungal activity was a phospholipide (lyso-fosfatidyl-etanolamine hexadecenic acid), but it was only found to have activity once, and when the results proved difficult to repeat the project was abandoned. A possible mechanism by which MF 626 is able to suppress *F. culmorum* can be to inject this phospholipide, thereby destabilising the membrane, hence making the fungi osmosis-sensitive. Microscopic observations indicate such a scenario, but not always! Deformations have been observed in parts of the mycelium rendering abnormally broad and swollen hyphae. In the growing tip of the hyphae, a kind of stunting has been observed (Figure 7). These observations were made when the 'cellfree' supernatant (after ultracentrifugation at 10000 rpm) had been fractionated through a solid phase C18-column, where acetonitrile (0, 40, 70 and 100%) was used as the mobile phase. After evaporation of the acetonitrile the fractions were tested for inhibition of spore germination (**II** & **III**). Throughout this procedure cells of MF 626 had survived and in the hydrophilic fractions it had this effect on the mycelium. The work to determine the mode of action of the biocontrol of this strain is not yet finished.

Figure 7 Deformations of F. culmorum hyphae (A) in presence of P. agglomerans strain MF 626, compared to healthy hyphae (B)



4.4 Regulation of biocontrol traits

It is somehow difficult to separate different types of regulatory systems since there is a lot of "crosstalk" among them. Often, but not always, there are equivalent systems in human- and plant-pathogenic bacteria, and those genes have often other names than the ones found in environmental bacteria. (A fact that certainly does

not make it easy to understand this area of abbreviations of gene names, protein names, and known and unknown factors that seems to regulate each other in surprisingly many ways, back and forth).

4.4.1 Global regulation

Many secondary metabolites are regulated through the GacS/GacA two-component system, where 'S' denotes a sensor kinase that is activated by an external or internal signal. The signals that activates the sensor kinase are for instance; pH, temperature, osmolarity or bacterial produced density dependent signals. The autophosphorolated sensor protein, (GacS) activates another protein by phosphorylation, which turns it to an activator (GacA) of gene transcription (Ligon *et al.* 2000; reviewed by Heeb & Haas, 2001). This regulatory system has been identified pathogenic bacteria and in BCAs. GacS/GacA is involved in the regulation of many different bacterial traits such as; motility, formation of pili, pathogenicity, siderophore production, secondary metabolite production and it also positively controls the production of N-acyl homoserine lactones (AHLs, se below) (Heeb & Haas, 2001). GacS/GacA deficient colonies are often seen as spontaneous mutations characterised by lack of colony organisation, and a general decrease in vitality (Duffy & Defago, 2000). Examples of antifungal metabolites regulated by this system are pyrrolnitrin (Gaffney *et al.* 1994; Ligon *et al.* 2000); DDR (Wright *et al.* 2003); phenazine (Chancey *et al.* 2002) DAPG, pyoluteorin, resorcinol (references in Heeb & Haas, 2001 and Chancey *et al.* 2002) 2-acetamidophenol (Slininger *et al.* 2000).

Two other examples of global regulation are the FNR/ANR (anaerobic regulation called FNR in *E. coli* and ANR in *Pseudomonas* spp.) (Blumer & Haas, 2000), and the Fur-repressor protein, both regulated by binding and releasing of iron (Neilands, 1990; Kiley & Beinert, 1999). Low oxygen levels would in facultative anaerobic bacteria start-off a number of events altering the respiration pattern from respiratory to fermentative, but also causes more limited changes in some bacteria. The production of HCN is up-regulated either by a low oxygen pressure or a high iron level, (Blumer & Haas, 2000). The authors demonstrated that the expression of the *hcn*-gene (coding for HCN-synthetase) was regulated by the anaerobic sensor protein (ANR). The FNR protein contains two $[4Fe\text{-}4S]^{2+}$ clusters in its active form, and is inactivated by oxygen, which reduces the clusters to $[2Fe\text{-}2S]^{2+}$ (Kiley & Beinert, 1999), and the authors (Blumer & Haas, 2000) suggest that HCN production is regulated in a similar manner by ANR, rather than via the GacS/GacA regulation as has previously been suggested.

4.4.2 Temperature-dependent regulation

When searching for references on psychrotrophic or psychrophilic bacteria (bacteria that thrives at low temperatures, see Glossary), two main research areas appear; bacteria spoiling diary products, and bacteria from arctic zones. There are some examples of psychrotrophic bacteria from other research areas as well (see below). The reason for the apparent scarcity in the scientific literature could be that those working on low-temperature tolerant bacteria in other areas, are not using the term "psychrotrophic" bacteria, since this term is might be confused with

psychrophilic, or for weaknesses in definitions of the terms (see Glossary). For a review of biochemical differences between psychrotrophs and psychrophils I recommend Russell (1990). In the medical area the term "psychrotroph" is (incorrectly) used for all bacteria that have a growth optimum below 30°C (Prescott *et al.* 1993), which for outdoor conditions in most parts of the world would be considered quite warm. In the temperate zones of the world, where great temperature-shifts during spring and autumn are common, it is almost a prerequisite that potential BCAs have the ability to grow also at low temperatures.

There are quite a lot of reports of thermo-regulated enzyme synthesis described for pseudomonads that spoil diary products (Zachariah & Liston, 1975; Hellio *et al*. 1993; Burini *et al*. 1994; Guillou & Guespin-Michel, 1996; Laurent *et al.* 2000). One psychrophilic *P. fluorescens*, strain MF0, isolated from raw milk, has been much studied, and a critical temperature for shift in the gene expression has been established (Guillou & Guespin-Michel, 1996). At 17.5°C this strain has an optimum for production of several extracellular enzymes like; lipase, acid phosphatase, and protease (Hellio *et al*. 1993; Burini *et al*. 1994). In a screening for thermoregulated genes in the MF0 strain, this group found that nearly 40% (!) of the genes were thermoregulated (Regeard *et al.* 2000). There are also reports describing temperature-responsive genetic loci in plant pathogens like *P. syringae* pv. *glycinea* (Ullrich *et al.* 2000). The expression of genes coding for synthesis of the pathogenicity factor, the phytotoxin coronatin, was shown be induced at 18°C. In *Cryseomonas luteola,* pectate lyase, but not cellulase seemed to be temperature-regulated (Laurent *et al.* 2000). Endophytic bacteria were more *in vitro* inhibitory to *Agrobacterium vitis* when they had been cultivated at 15°C compared to a cultivation temperature of 30°C, indicating that the expression of the active metabolites were under temperature-dependent control (Dickie & Bell, 1995). It has been shown that the antifungal compound DAPG was produced in varying amounts at different temperatures, with the highest amount produced at 12°C (Shahanan *et al.* 1992). The psychrotrophic *P. fluorescens* strain ANP15 produces more pyoverdine at 12°C than at 19°C, the optimum temperature for growth of this strain. Likewise does the *P. aeruginosa* strain 7NSK produce more pyoverdine at a temperature (19°C) below its growth optimum temperature (28°C) (Seong *et al.* 1991). The temperature-dependent regulation of this siderophore has previously been reported (Garibaldi, 1971). In an experiment done by Slininger & Shea-Wilbur (1995), they obtained maximum production of phenazine at the lowest temperature tested (25°C), while the growth optimum was around 30°C. The above-mentioned observations indicate that some temperature-sensitive signal(s) are produced. The signal(s) would be different from the inducer of the so called cold-shock proteins which have been defined in several bacterial species and are accumulating in a linear mode correlated to a decreasing temperature (Hebraud *et al.* 1994), while the production of the mentioned substances have a peak of a maximum production at a certain temperature. A candidate system for this type of regulation was suggested by Suzuki *et al*. (2000). They identified two histidine kinases (Hik33 and Hik 19), where one likely is to be a membrane-bound sensor and the other a signal transducer.

4.4.3 Other regulatory factors

Stress of various kinds has traditionally been considered to trigger the production of secondary metabolites, and the temperature-dependent regulation discussed in the previous section could of course also be defined as a stress-related trigger. Other such stresses that have been suggested are a low phosphate level, which stimulates production of pyocyanin, phenazine and HCN (Leisinger & Margraff, 1979). The peptide-part of iron-chelating pyoverdines is produced by a cytoplasmatic multi-enzyme including an iron repressed peptidsynthetase (Georges & Meyer, 1995). In contrast, the synthesis of the siderophore Pseudobactin 384 is regulated by iron directly at the transcriptional level (Weisbeek *et al.* 1990).

Quorum sensing (QS) or density-dependent regulation of gene expression was first described in Gram-negative BCAs by Pierson III *et al.* (1994). The population density is sensed via the production of N-acyl homoserine lactones (AHLs), which are small diffusible molecules, which can be species-specific, but are all chemically related. When the AHLs reaches a certain threshold concentration they interact with a signal generator protein and this complex can in its turn activate transcription of certain genes (Laue *et al.* 2000). Once the QS-dependent signal is produced it communicates with the GacS/GacA system, described above. The AHL-signal does not always seem to be very specific, since it was demonstrated that bacterial populations of completely different origin had the ability to induce phenazine production in AHL-deficient mutants of *P. aureofaciens* 30-84 (Pierson *et al.* 1998). See also 2.1.2 and 5.2.2.

5 Some philosophic remarks

5.1 Survival of the fittest

As microbiologists, we sometimes have a tendency to regard bacterial isolates as individuals with specific, and stable, traits whereas they are in fact populations composed of several billion individuals harbouring a greater or lesser extent of genetic variation. When the environment changes, some of these individuals will have an advantage over some others, thus taking a more dominant position in the population. This is, of course, nothing new but often we behave as if this phenomenon is something that has to do with evolution and has little to do with our everyday work in the lab. Sometimes it can be interesting to take a step backwards and try to get some distance to things "everyone" takes for granted. Take for instance the tradition of keeping bacteria, and fungi for that matter, in pure cultures. As a microbiologist, it is almost embarrassing if someone you have given a culture claims that it was impure. It is a criticism of your professionalism. But one of the most known and acknowledged discoveries in microbiology was done just because of such a "contaminated" culture. I do not propagate for impure cultures *per se*, this is only an example of one of all the things we do, just because we always have done it...

Once a "wild" bacterium is taken into the lab and cultivated on lab media the selection-process towards a "lab-strain" has started, and in this process a lot of valuable properties might be lost. Growing the isolate on rich medium in a stable temperature can easily give rise to "spoiled" isolates, and there is a greater risk for it each time we transmit the isolate to new fresh plates, especially if we use the single colony purification method we have been thought as students. I think that in many cases it can be inappropriate to consider all the colonies that are growing on a plate as a clone. What we observe on a plate is a lab-adapted population, which is likely to have less fitness in a natural environment. However, within this population there is also a dormant potential of greater or lesser adaptability. It is difficult to really understand the strategies for survival of an organism so different from ourselves. The total number of human beings living on this planet is less than 10^7. Even though the human genome is much bigger and more variable this comparison could perhaps give a hint on the genetic variation on a plate with a bacterial isolate. As another comparison, it took the first humanoid *Australopithecus aphaeresis* approximately 2.5 million years to evolve into the human known as *Homo sapiens.* A bacterial isolate grown in continuous culture could undergo a corresponding evolution in less than five years, assuming the evolution is based on mutations and correlated to number of generations. This is of course a simplification of matters, but the purpose of the comparison was to illustrate that bacteria have a profoundly different time perspective, which I think is difficult for us to fully interpret.

In my work with the coding of colony morphology traits I often observed strange colony abnormalities, which could not really be explained as being contaminants. The most common change was loss of pigments, and in older cultures where the nutrition was exhausted, there was often a kind of phase-change in the colony organisation, where lobes of actively growing cells came out from the drying "mother colony". One exiting observation is that in some isolates there seem to be a kind of "symbionts" which is dependent on the association to the other bacteria, that is, it is impossible to separate this other type, which can be quite distinct in appearance, from the original isolate. Observations regarding the variation in "pure cultures" has also been reported several times in the literature. In a culture of a *P. fluorescens*, used for biocontrol of *Rhizoctonia solani*, Gaffney *et al.* (1994) observed bacterial colonies with different morphology appearing after only one week in room temperature. These colonies proved to be pleiotrophic mutants, which had lost several different abilities essential for biocontrol like, production of chitinase, pyrrolnitrin and HCN. After characterisation of a DNA fragment in the wild-type strain, and insertion of this fragment into the mutants, both the colony morphology and the biocontrol activity were restored. This was one of the first report of spontaneous GacS/GacA mutants and has thereafter been observed to occur as a quite common event (Duffy & Defago, 2000), which is also reversible Achouak *et al*. (2000), who described the new species *Pseudomonas brassicacearum,* identified a fluorescent colony type, which appeared at the edges of the original non-fluorescing type at late stationary phase when grown on Kings' medium B. They found that this new type also had lost several traits like, ability to hydrolyse gelatine and Tween 80, and to reduce nitrate. These two examples illustrate the ability of r-strategists, like bacteria, to adapt very quickly to a

changed environment. If we "spoil" our lab-strains too much they might find it harder to adapt back to something less pleasant like the battle zone in the rhizosphere environment.

5.2 What is a bacterium?

5.2.1 What is a bacterial species?

Microbiologists know that what we normally consider a species, doesn't really apply to bacteria, so why do we insist on trying to squeeze them in? I suppose it is in the human nature trying to categorise and systematise everything. With the new molecular tools that are rapidly developing, new species of bacteria are described in a confusing speed (Elomari *et al.* 1995; Coroler *et al.* 1996; Elomari *et al.* 1996; Verhille *et al.* 1999; Achouak *et al.* 2000; Andersen *et al.* 2000; etc.). The number of pseudomonads has increased with approximately 40 species since 1990, and before that the total number of species was around 100 (Euzéby, 2002). The bacterial strains in this work that were subjected to identification attempts turned out to belong to different species depending on which method that was used (**II**). Seldom there was an identification without several characters that normally wasn't connected with the species in concern. For instance is MF 30 an atypical *P. veronii*, MF 626 an atypical *P. agglomerans*, the identity of the IODS still is *Pseudomonas* spp., and so on. The BCA *P. chlororaphis* MA 342, has much more in common with the other 56 IODS than with the characters that defines this species (Table 5 in **II**). In a review by Cohan (2002) the species concept is discussed, and he has some suggestion on how to deal with this matter. Since the variation within named species of bacteria is greater than within most eukaryotic genera, it would be justified to have a third name. This is already applied with for instance *P. syringae* which almost always has also a patovar name. This third name should, according to Cohan, be labelled "ecotypus" and could thus be used also for non-pathogenic bacteria. This new groupings would better fit the commonly accepted species criterion, which is used for all living organisms. In this system the IODS would maybe be a natural group named *Pseudomonas mandelii* e. *guttatus*.

5.2.2 Are bacteria unicellular?

In a review by Shapiro (1998), the bacterial population in for instance a micro-colony on a root is considered to be a multicellular organism, rather than autonomous cells. The ability to shift between unicellular and multicellular stages is a well known feature among the Myxobacteria, and in recent times many of the mechanisms that regulate their cell organisation have been clarified. The discovery of the N-acyl homoserine lactones (AHLs) known as "quorum sensing"-signals have created a more positive attitude to the idea of multicellularity as a common strategy of bacteria to compete, use resources, and defend themselves more efficiently. In my work with the morphological classification of colonies, I was often fascinated by the organised patterns observed on the colonies (see Figure 3). The shift from unicellularity to multicellularity in Myxobacteria is dependent on nutritional supplies. When nutrition is limiting they organise themselves to form

fruiting bodies that produce resting spores, which are extremely resistant and can be viable up to ten years (Prescott *et al.* 1993). Apart from the fruiting body formation, the ecology of the Myxobacteria is quite similar to the ecology of the pseudomonads. In Prescott *et al.* (1993) Myxobacteria are described with these words "... gram-negative, aerobic soil bacteria ... rods ... secrete an array of digestive enzymes ... secrete antibiotics ... chemoheterotrophs with respiratory metabolism", all characters usually listed for non-pathogenic pseudomonads (Stolp & Gadkari, 1981).

In my observations of bacterial colonies it seems as if the pseudomonads have an opposite trigger to shift from unicellularity to multicellularity, that is, they form colonies when nutrition is abundant. Another aspect of bacterial life is the temporal development of an individual cell, related to the development of a population in form of a colony, a biofilm or aggregates in liquid. Hypothetically, there could be similarities between how honey bees are organised. Honey bees have an age-dependent organisation, where each individual of the working bees, at the end of their life has had all the different tasks that occur in the bee hive. The shifts between the different tasks are achieved with changes in the food composition. Also the queen egg is just another egg, but by feeding the larvae with a particular food, queen jelly, she develops into a queen (Hansson, 1980). Based on my observations and inspired by the literature (mostly the scientific one, actually) I tried to imagine how root-colonising bacteria do, and the result became this little story.

5.2.3 The story about little Pseudo Monas and her big family[1]

Little Pseudo was born on a wheat seed and it was dark and cold. She was surrounded by many ancestors that had finished their existence and now were giving back their energy to the collective. Pseudo was full with life and loved to swim around and experience all the things that she sensed through her receptors. Her best friend was Fluore and the two of them were swimming together eating sugar and life was smiling at two young bacteria, fifteen minutes old. Then things changed, there was no longer any food and little Pseudo and her friend felt hunger. It was a terrible feeling and it made them try to eat whatever came in their way. They did find things to eat, acids and such hard-chewed things, not at all as the sugars they had eaten before. But then something wonderful happened, Fluore found a sugar and told Pseudo that it was coming from below. Instantly, they turned in that direction, and Yes! Pseudo also found one. They swam towards the sugar and knew they were on the right track since they found more and more. As they swam they passed many of their sisters that had already settled and had started to divide. It also awakened this desire in them, to find a good place, getting settled and making a colony. The both friends had lived for one hour. Pseudo felt tired of swimming and had encountered a kind of food that she had never tasted before. It had a strange effect on her and now the urge to get settled was stronger still. She felt that many of her relatives were sitting together somewhere behind

[1] This story is a product of my imagination, even though it is based on some known facts concerning bacterial colonisation of roots.

the epidermis cells of the root surface, and she wanted to join them. She was calling for Fluore, but then she saw that she had already attached. Pseudo said goodbye to her friend and wished her good luck. Fluore seemed happy, and it was a good spot that she had found for making her colony. Pseudo felt the changes in Fluore and realised that there were so many things that were going to happen once she was getting attached. Hurriedly, she swam towards the inside of the root, and again this strange food came to her. Again she sensed the song of her relatives, it was many more of them now, in this place it was possible to divide rapidly it seemed. Pseudo felt that her flagella were about to be dropped and that pili were growing. She extended some of the pili and glued them to the plant cells' surface. The flagella were itching so she twitched to get rid of them. The new food in this place allowed her to make new molecules and she enjoyed it like an artist creating something beautiful. Some of the new molecules she weaved together with the plants' extensions and finally she was attached. She felt adult and responsible and it was a good feeling, after all she was two hours, so it was about time to get settled and start dividing. She wanted her little dividings to have a good start in life, so she made a lot of enzymes and sent them out in the surroundings to do their work. Then she started to divide! It was a wonderful feeling, she was not alone anymore. She was one and she was many, and she organised herself in a beautiful colony, round and shimmering in all colours of the rainbow. She felt so pleased that she started to sing. The song went stronger and stronger and her tune joined the tune of her relatives. These were the happy times. But life was about to change. Pseudo felt a strange taste and at the same time the plant sent an alert that an intruder was approaching. Her relatives in the neighbourhood sent the signal announcing that now it was time to start producing armour. In fact Pseudo had already produced some, since she was a responsible bacterium, and here in the centre of the colony the food was not so abundant anymore. She was supported of course, by her dividings, but as an old bacterium it was also one of her tasks to make some armour, just in case they were going to be needed. Now she transported the armour out to the surface and felt that the cell she once was didn't live anymore, nor did the first dividings. But the intruder had caused much damage to the plant on its way down here, and that meant also food for her dividings. The song was not heard anymore in that neighbourhood, but if she listened carefully she could sense it from further down below. Through the receptors of her youngest dividings she sensed the melting remainings of the cell she once was, and again she could experience the young happiness of swimming around and eating sugar.

THE END.

6. Conclusions

- Psychrotrophic rhizosphere bacteria that suppress the development of wheat-seedling blight, caused by *Fusarium* species, and the development of snow mould in wheat, caused by *Microdochium nivale,* were found.
- Most bacterial isolates that suppressed diseases caused by *F. culmorum* also suppressed diseases caused by *M. nivale* and other species in the fusarium complex that are pathogenic to cereals.
- *Pseudomonas* sp. strain MF 30 was disease-suppressive in two completely different host-pathogen systems.
- A greenhouse bioassay with high correlation to results obtained under field conditions (r^2 0.72) was developed and used for screening.
- An early involvement of field experiments is crucial to the outcome of the screening work.
- The method used for isolation has an impact on the proportion of disease-suppressive isolates found.
- In this work, a higher proportion of disease-suppressive isolates was found when the plants used for isolation belonged to the Brassicaceae family and when the samples were taken in the spring. A low content of soil and water in the samples used for isolation also had a propitious effect on the proportion of BCAs, as did isolation on the specially designed soil-extract medium.
- The commonly used biochemical methods of bacterial identification (BIOLOG, API20NE, FAME, and PhenePlate) did not provide an unequivocal identification of most of the isolates tested in this study.
- The bacterial colony morphology can provide information on biochemical bacterial traits also when bacteria are grown on complex media.
- For bacterial BCAs the most studied mode of action is antibiosis. For the future of biocontrol in an economical perspective, it is my impression that it would be preferable to concentrate the efforts in search for and development of bacteria using other modes of action, such as induced resistance, niche exclusion, and other, still unexplored, modes of action.
- Since biocontrol is an introduction of one or few organisms to a complex system of interactions, it is difficult to draw conclusions that apply to field conditions, from results obtained in sterile and gnotobiotic laboratory experiments, before these observations have been confirmed *in vivo*.
- A general conclusion of this work and the literature review is that a high level of adaptability to different environments is a common feature among rhizosphere bacteria that are successful BCAs. Thus, a BCA is a bacterial generalist.

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"The oracle told me..." - "She told you exactly what you needed to hear, that's all. Neo, sooner or later you are going to realise, just as I did, there's a difference between knowing the path...
... and walking the path"

Neo and Morpheus in 'The Matrix',
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