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Edited by: Birger Koopmann, Simon Oxley, Andres Schützendübel and Andreas von Tiedemann

Ramularia collo-cygni

A New Disease and Challenge in Barley Production

Proceedings

First European Ramularia Workshop

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Birger Koopmann, Simon Oxley,
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Editors:

Dr. Birger Koopmann [1]
Dr. Simon Oxley [2]
Dr. Andres Schützendübel [1]
Prof. Dr. Andreas von Tiedemann [1]

[1]

Department of Crop Sciences
Division of Plant Pathology and Crop Protection
University of Göttingen
Grisebachstr. 6
D-37077 Göttingen

Phone: +49 (0)551 393700
Fax: +49 (0)551 394187
URL: http://wwwuser.gwdg.de/~instphyt/

[2]

Scottish Agricultural College (SAC)
King's Buildings,
West Mains Road,
Edinburgh EH9 3JG

Phone: +44 (0) 131 535 4094
Fax: +44 (0) 131 535 4144
URL: http://www.sac.ac.uk/

Ramularia Workshop Website: http://wwwuser.gwdg.de/~rcc/index.htm

Ramularia web page, Federal Biological Research Centre for Agriculture and Forestry, Germany:
http://www.bba.bund.de/cln_044/nn_804436/EN/Aktuelles/Ramularia/Ramualaria__Inhalt.html__nnn=true

Ramularia web page, SAC: http://www.sac.ac.uk/consultancy/cropclinic/clinic/diseases/ramularia

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Preface

The First European Ramularia Workshop was well recognized by the scientific community, chemical and breeding companies. We were overwhelmed by the unexpected high number of people interested in the meeting. A total of 71 participants from 12 European countries (Austria, Czech Republic, Denmark, France, Germany, Netherlands, Norway, Poland, Russia, Switzerland, Sweden and United Kingdom) attended the workshop, indicating the importance which the disease is gaining all over Europe. During the workshop 21 presentations were given providing information about history of research, epidemiology, pathogenicity, resistance and chemical control of Ramularia. These contributions were summarized in a book of abstracts, which is available for download from the website http://wwwuser.gwdg.de/~rcc/index.htm. Authors were also asked to provide full papers of their workshop contribution. With the electronic publication of this proceeding, we offer you the most recent information on Ramularia. We warmly thank all authors who worked hard on providing a full paper, which will become a valuable source of information.

We apologise for the delay of publication due to severe computer problems within our editing team. We hope that all of you will find this proceeding helpful for your own work.

The name `First European Ramularia Workshop´ suggests that there will be a following workshop. A next workshop is planned to be held in a three year interval. Simon Oxley kindly offered to host the `Second European Ramularia Workshop´ in 2009 at the Scottish Agricultural College in Edinburgh, Scotland. The details of this workshop will be circulated by email when it is decided when it will take place. For those who are interested and are not listed on the participants mailing list (or had a change of address) should inform Simon Oxley (email: Simon.Oxley@sac.ac.uk) to put her/his name on the list.

Andreas von Tiedemann, Simon Oxley, Andres Schützendübel and Birger Koopmann

Introduction

The history of research into Ramularia leaf spot on barley

Edelgard Sachs

Federal Biological Research Centre for Agriculture and Forestry, Kleinmachnow Branch, Germany

Abstract: The historical overview of research into Ramularia leaf spot [RLS] starts with 1893 and ends in the presence. Research results from Europe but also New Zealand, Mexico and the Argentine are presented. The contribution focuses on the own phytopathological work at the Federal Biological Research Centre for Agriculture and Forestry. Intensive research into the disease and its pathogen, *Ramularia collo-cygni* [*Rcc*], has been done in the last 20 years. First, attention was especially paid to the occurrence, distribution, ethiology and morphology, later to taxonomy, biology, symptomatology, epidemiology and biochemistry. Although there is still more or less need to deal with these subjects, research has recently concentrated on practical aspects like importance and chemical control of the disease, host resistance and pathogen variability. Finally, the prospect for further research is given.

Key words: History, *Ramularia collo-cygni,* disease, barley, occurrence, distribution, ethiology, diagnosis, epidemiology, biochemistry, toxins, chemical control, host resistance

It is very peculiar that RLS was described already 100 years ago, but relatively little is known about the disease. Cavara described the pathogen first for Northern Italy as *Ophiocladium hordei* Cavara (1893). In the mid 20ies of the last century, Jørstad (1930) reported on the occurrence of the disease in Norway. This was followed by a long time without any reports. In 1980, a barley breeder in Norway found so far unknown symptoms on barley, but could not identify them (Salamati *et al*., 2004). At this time, unknown disease symptoms on barley were also found on both islands of New Zealand and false identified as physiological spots. In 1983, these spots were identified as *Ovularia hordei* (Cavara) Sprague, a synonym of *Rcc* (Cromey *et al*., 2004). In New Zealand, the disease is called Ramularia leaf and awn spot. In 1985, Huss finally noted the strong occurrence of unknown leaf spots on barley in Austria. He attributed it to the fungus described by Cavara and made a detailed description of it (Huss *et al*., 1987). This was the start of intensive research into RLS in Upper Austria, which has not stopped so far. Huss called the disease speckle disease because of the speckle-like symptoms. Shortly after, small notices on the occurrence of the same fungus on barley were also published in Switzerland (Brönnimann, 1988). Another important event was that Sutton and Waller identified the disease on triticale in Mexico and re-named it as *Ramularia collo-cygni* (Sutton & Waller, 1988). The name of the species *collo-cygni* is derived from the special swan neck shape of the conidiophores (collum = neck, cygnus = swan). In 1992, Huss published a sensational documentation of raster electron microscopic photos on the morphology of the asexual structures of the pathogen.

A milestone in the history of *Rcc* research was the publication of the monograph of *Ramularia* and allied genera of Braun in 1998. He provided a detailed description, illustration, a summary of hosts and distribution and discussion of the taxonomy of this species. According to Braun, it has a unique morphology within the genus *Ramularia*.

In 1997, I obtained a sample of barley from Lower Austria. It draws my attention to the disease. The consignor informed that there were different opinions on the pathogen. Some phytopathologists thought that these spots are physiological leaf spots [PLS], but Huss was of the opinion that the symptoms were caused by *Rcc*. My microscopic investigation confirmed Huss. Then I contacted him. This was the beginning of a fruitful co-operation. The dispute on whether the leaf spots are caused by the fungus *Rcc* or whether they are the non-parasitic leaf spots called PLS had lasted for several years among phytopathologists and barley breeders and had started strong controversies. Even today not all critics are likely to be convinced of the significance of the fungus as pathogen of RLS. Since 1997, we in the Kleinmachnow Branch have tested all barley samples we obtained for an infection with *Rcc*. At first we clearly identified samples from Middle Franconia to be infected (Sachs *et al.*, 1998), but also a herbarium barley leaf sample collected in Upper Bavaria in 1992. The identification of the disease on herbarium samples on the basis of still visible conidiophores is interesting because herbarium samples from other regions could prove when the disease has actually been present there. In the succeeding years infection was found in Ireland (Sachs *et al.*, 1998), Scotland (Jahn *et al.,* 1999) the Czech Republic (Amelung *et al.*, 1999), Norway (Salamati *et al.*, 2003), Denmark (Sachs and Huss, 2004), France (Sachs 2004b) and Belgium (Balz, pers. comm., 2005). Samples obtained from Uruguay and Argentina (Sachs, 2002) showed also strong infection.

The heavy infection with *Rcc* in Scotland, which we identified (Jahn *et al.*, 1999), led to the 3-year Scottish research project "Development of a rationale to identify the causal agent of necrotic lesions in spring barley and identify control mechanisms". The project was headed by Oxley from the Scottish Agricultural College, Edinburgh and integrated further British phytopathologists, but also phytopathologists from Ireland, Norway, Austria and Germany. The project activities supported the co-operation of the participating scientists and the intensive discussion of the various leaf spots on barley, especially those caused by RLS and PLS (Sachs and Obst, 2000). Diagnosis and the fulfilment of the Koch's principle to identify the pathogenicity of *Rcc* (Huss and Sachs, 1998; Salamati, 2004; Frei, 2004) were subject to detailed discussion, and control and susceptibility of cultivars played an important role. Another interesting subject was the isolation and cultivation of the fungus on artificial substrate of *Rcc* as basis of the future resistance tests (Frei and Gindrat, 2000; Sachs and Huss, 2004; O'Sullivan, 2004). The establishment of a collection of strains of the fungus proved to be an important prerequisite of PCR by Havis (Havis *et al.*, 2004).

Later we found out that the Ramularia toxin turns red. This knowledge has been a key to future research. It allowed to develop a diagnostic quick test (Tschöpe and Sachs, 2001). It uses very simple laboratory equipment to test larger quantities of samples for *Rcc* and other causes of leaf spots within 2 days. Only a short time is necessary to get familiar with the test and thus to be able to distinguish between *Rcc* and PLS leaf spots and to distinguish mildew necroses and net blotch. The tests allowed to gain experience in symptomatology which is of enormous value for field work (Sachs, 2004a). Balz and v. Tiedemann (2004a) developed an ELISA test to identify the disease before first symptoms are visible. The quick test we developed was the basis to conduct a monitoring throughout Germany (Sachs, 2004a). In the beginning, the South of Germany showed stronger infection, but in the following years infection increased also in the Middle and North of Germany. Even the drier East of Germany showed infections (Kreye, pers. comm., 2005). Monitorings carried out later did not show any more a regional focus (Balz and v. Tiedemann, 2004b). Monitorings were also carried out in Argentina (Kiehr *et al.*, 2002), Austria (Formeyer *et al*, 2004), Norway (Salamati *et al.*, 2004), and the Czech Republic (Minarikova *et al.*, 2004).

The diagnostic quick test allowed comprehensive investigation into epidemiology (Sachs, 2004a). It was found out that first infection occurs on young winter barley on warm autumn days. The pathogen hibernates on the oldest leaves of the plants. In the following spring it starts spreading across the leaf layers with the beginning of the growing season until it reaches the awns. Mass production of conidia often starts in Germany at the flowering stage if the weather is wet at this time. Flag leaves and awns are sometimes heavily infected. From winter barley the disease spreads to spring barley, but also to other cereals, grasses and maize. In Germany, volunteer barley is of great importance as intermediate host. Dew plays a great role for infection (Formayer *et al.*, 2004). According to new investigations by Minihofer (2003) the pathogen is able to sporulate and to infest also in winter.

Miethbauer *et al.* (2004) identified the red dye formed by *Rcc* as Ramularia toxin rubellin D and studied the molecular structure. They found out that the anthrachinon derivative rubellin D isolated from the cultivation substrate of the fungus causes the same symptoms on barley as the pathogen. Further investigations by Heiser *et al.* (2004) have recently shown that the fungus toxin contains also rubellins A, B and C which were also identified in the Mycosphaerella toxin. This supports the result of Crous *et al.* (2000) who carried out a molecular analysis of *Rcc* and showed that the fungus belongs to the monophyletic Mycosphaerella cluster. This means that it belongs to the ascomycetic order *Mycosphaerella* if the teleomorph of *Rcc* is found some day. Last time the biochemical work on the phytodynamic activity of rubellin D were completed (Heiser *et al.,* 2003).

Another milestone in the history of Ramularia leaf spot research was the 2nd International Workshop on Barley Leaf Spots carried out in Syria in 2002. For the first time *Rcc* appeared on stage and was of great interest. Nearly all scientists working in the field presented their results. As a result Sachs and Huss (2003) established a homepage that informs on all knowledge available up to 2002.

The last five years saw further research into the control of the disease (Huss, 2000; Burke *et al.*, 2001; Salamati *et al.*, 2004, Cromey *et al.*, 2004; Greif, 2004; Pinnschmidt and Hofmøller, 2003, Balz and v. Tiedemann, 2004b). It revealed that strobilurine-containing fungicides are most effective. There are different opinions on the application time. Cromey (2004) recommends very early application, Balz and v. Tiedemann (2004b) recommend to carry out application as late as possible. Data on additional yield as compared to the untreated control differ considerably. It might be about 10% to 15%. There is no fungicide approved for Germany, but negotiations will soon be finished. It is very likely that the combination fungicide Amistar-Opti containing azoxistrobin and chlorthalonil by the Syngenta company will be registered this year.

The importance of RLS and of its control raised the question of forecasting. Cromey *et al.* (2004) supposes that the disease can hardly be forecast due to its strong deviation in occurrence. Norway, however, has started work on forecasting together with the meteorological service (Salamati, pers. comm.). As occurrence and leaf wetness duration are closely correlated (Formeyer *et al.*, 2004), forecasting has a good chance.

As the disease has obviously spread and as it has been realized as a serious threat to barley yields, several countries have made preparations for resistance breeding. So, cultivars and strains were checked for susceptibility (Cromey *et al.*, 2004; Sachs and Huss, 2003; Salamati, 2004; Balz and v. Tiedemann, 2004b; Pinnschmidt and Hofmøller, 2004). The cultivars showed differences. This is a basis for resistance breeding. Austria (Bistrich, pers. comm.) and the Skandinavian countries Denmark, Norway and Sweden (Salamati, pers. comm.) started resistance breeding on barley against RLS.

Field trials, however, showed that it is very difficult to identify *Rcc* resistance of a cultivar under field conditions. In New Zealand, strong net blotch made it more difficult

(Cromey *et al.*, 2004). In Norway, the cultivars showed a negative correlation between Rhynchosporium and RLS. Austria had a negative correlation between dwarf leaf rust and *Rcc* (Sachs and Huss, 2003). Field trials carried out by Huss (Sachs and Huss, 2003) showed that field trials may lead to the assumption that late-ripening cultivars might be less susceptible. For this reason, tests should be carried out under controlled conditions with artificial inoculation of a defined inoculum in an environment controlled glasshouse or climatic chamber. This requires defined conidia suspensions and well identifiable disease symptoms. Our trials (Sachs and Huss, 2003) and those of O'Sullivan (2004) showed that these conditions are hardly to guarantee. Norway, however, obtained disease symptoms in glasshouse conditions similar to those in the field (Salamati, pers. comm.). Huss continued his work on the host range of *Rcc* and found strong infection with *Rcc* in wheat and oats in Austria in 2005 (Huss *et al.*, 2005).

Meanwhile, RLS has appeared in some German dictionaries (Schöber-Butin *et al.*, 1999; Obst and Gehring, 2002). German breeders, plant protection experts and representatives of the chemical industry were trained for the identification of the disease and its pathogen in 2004 (Sachs, 2004c). This was an important contribution to general information on the disease.

This is far from answering all questions connected with RLS. We do not know why the disease is spreading so rapidly. We have insufficient knowledge of the environmental influence on the disease's spread. There is knowledge of host resistance, but the cause is not known. Furthermore, a procedure to test resistance has to be developed. We do not have enough knowledge of the influence of cultural practices on infection processes. Furthermore, we lack knowledge of pathogen variability. We guess whether the fungus might attack new host plants and which ones. As to taxonomy we still have not found the teleomorph of the fungus. But the question of all questions is: What effect does *Rcc* have on the quality of beer and whisky? And it would be ridiculous if it were not possible to raise funds for the subject.

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Epidemiology

Ramularia collo-cygni on spring barley, an overview of its biology and epidemiology

Saideh Salamati[1) and Lars Reitan[2)
[1) *Midt-Norsk Plantevern AS and Bioforsk Midt-Norge, Kvithamar Research Centre, N-7500 Stjørdal, Norway.* [2) *Graminor AS, branch Holthe, N-7650 Verdal, Norway.*

Abstract: *Ramularia collo-cygni* (Rcc) is now gaining more attention as a serious pathogen of barley. Even though several scientists have considered it the main cause of the typical necrotic spotting of barley, still as late as 2004 obvious doubts exist on its being a real pathogen (Heiser *et al*, 2004). A great deal of uncertainties is related to the fact that hitherto the attempts for artificial inoculation in controlled environments have not given the exact disease symptoms. Central Norway has cold and humid summers. The typical leaf spotting which later received the name Ramularia Leaf Spot of Barley (RLSB) was observed in early 1980s in this area. Since 1998 we have registered the disease every year. After about eight years working with RLSB, we have acquired some understanding about its causal agent, Rcc. In April 2005 a detailed experiment was carried out in green house and 3 barley varieties were artificially inoculated with Rcc. Typical disease symptom was registered on the most susceptible variety (Lavrans) four weeks after inoculation. Later the pathogen was isolated from all three varieties with and without obvious disease symptom. In central Norway RLSB was the main disease of spring barley in 2005. Rcc developed similar type of spotting on oat and couch-grass (*Elytrigia repens*) as well. Over 230 Rcc isolates were collected (200 from barley and 30 from oat). We have looked at their sporulation abilities, growth habits on different cultural media and colour differences.

In connection with the decision support system VIPS (Bioforsk) we looked at the historical weather data in central Norway. Humid weather conditions at the beginning of barley growth which in central Norway occurs around the first 10 days of June is highly correlated with the final RLSB severity, suggesting an early infection. We recognize the disease much later often at or after flowering. Based on an overview of all the results we will hypothesise a relationship between the pathogen and the barley plant, this to understand some of the disease's epidemiology.

Key words: *Hordeum vulgare* L., *Ramularia collo-cygni* Sutton & Waller, biology, epidemiology.

Introduction

In early 80s in several parts of the world a typical type of spotting on barley leaves, sheaths and awns was recognized. It was characterized by abundant small brown speckles usually with a yellow halo occurring in most cases late in the growing season (figure 1). The spots were often confused with physiological spotting caused by abiotic factors. The rapid senescence of the leaves (premature ripening) causes economical damage to barley quality and quantity. Around mid 80s the fungus *Ramularia collo-cygni* Sutton &Waller (1988) was identified as the causal agent both in Europe (Huss *et al.*, 1987) and the New Zealand (Harvey, 1986). The disease has been recognized on other grass species in parts of Europe

(Huss, 2004) on wheat, oat, maize, couch grass (*Elytrigia repens*) as well as in Norway (oat and couch grass).

Figure 1. Typical symptoms of Ramularia Leaf spot of Barley.

In Norway Ramularia Leaf Spot of Barley (RLSB) was recognized in early 80s like several other European countries. The fungus was isolated for the first time from diseased spring barley leaves in 1999. RLSB occurs mainly in the central part of Norway which has wet and cold summers (average summer temperature and precipitation are 11° C and 500 mm, respectively). Since 1999, research on RLSB was carried out at Bioforsk Midt-Norge (Norwegian Institute for Agricultural and Environmental Research), Kvithamar Research Centre in the county North Trøndelag in collaboration with the Norwegian national breeding company Graminor AS. Our work is emphasised on *R. collo-cygni* biology in order to understand its epidemiology and develop a screening method for barley breeding material in controlled environment. RLSB has been an official part of our breeding programs since 2000. We have also looked at the disease's significance for barley yield and its components. The first steps toward developing a forecasting plan against this disease has been taken in collaboration with Bioforsk decision support system VIPS (Varsling Innen Plante Skadegjørere). In this paper we present a summary of the work carried out since 1999 that concerns the biology and the epidemiology of *R. collo-cygni*.

The work carried out by Heiser *et al.* (2003, 2004) and Miethbauer *et al.* (2003) have revolutionised the understanding of *R. collo-cygni* – barely interactions. A major part of our discussion is based on their work. Heiser *et al.* (2003) found that *R. collo-cygni* produces a variety of colour components in Czapek-Thom-Medium. The pattern of these substances was variable and seemed to depend on culture conditions. They demonstrated that these colour components were attributed to a group of anthraquinone metabolites and were mainly consisted of rubellin B and D. Heiser *et al.* (2004) concluded that *R. collo-cygni* produces photodynamically active toxins (rubellins) and induce peroxidation of unsaturated fatty acids and co-oxidation of pigments. Miethbauer *et al.* (2003) suggested that the orange colour in

young cultures is attributed to the production of rubellin B. Rubellin B is firstly biosynthesized and will be converted to the more polar (soluble) rubellin D (reddish violet component). They also demonstrated that most of the rubellin in infected tissue is rubellin B (not soluble *in planta*). The involvement of rubellins in the infection process by *R. collo-cygni* was ruled out by Miethbauer *et al.* since they were not considered as host-specific toxins. Later Heiser *et al.* (2004) proved that Rubellin D is a non-host specific phytotoxin inducing symptoms not only on barley, but also on tobacco leaves. In the same paper they hypothesised possibility of rubellins as pathogenicity factors in barley - *R. collo-cygni* pathosystem.

Material and methods

Isolation of R. collo-cygni

We rinse newly detached leaves with tap water for 15-30 minutes. Whole leaves are placed with the upper sides faced down to water agar in rectangular Petri dishes. The plates were placed under black light (near ultra violet light, NUVL, 30 cm distance from the light source) at 22 ° C. After 24-36 h bunches of white conidiophores bearing conidia are protruding from leaf stomata (at the brown spots) in parallel rows. Spores are collected by a sterile needle and placed on the surface of VJA containing 50 ppm kanamycin (200 ml Granini vegetable juice, 2 g Ca CO3, 2 ml KOH 5 %, and 20 g agar/l).

Growing R. collo-cygni *on culture and sporulation media*

For *in vitro* cultivation of *R. collo-cygni*, we have used Wheat Germ Agar (WGA, filtered decoction of 35 g wheat germ + 18 g agar/l), PDA, VJA and for its sporulation, VJA, barley leaf extract agar (BLSA, filtered decoction of 200 g fresh barley leaves, 18 g agar/l), SA (filtered decoction of 20 g barley straw, 18 g agar /l)), malt extract agar (MEA, 20 g malt extract, 20 g agar/l) and potato carrot agar (PCA, filtered decoction of 20 g carrot + 20 g potato, 20 g agar /l). After inoculation (a small piece of agar with mycelia is either placed directly on the surface of a new agar plate or it is shaken in 1 ml of sterile distilled water and then spread over the agar surface, excess liquid is then discarded), *R. collo-cygni* plates are placed in a growth cabinet with a simulated day, night regime. The temperature is 20 ºC during the light period and 12 ºC while dark. Cultures are illuminated by two sets of two florescent lamps and one black light tube installed on the sides of the cabinet. In 2005 we had one of the most severe attacks of RLSB. We were able to collect about 230 isolates of the fungus from 4 fields in central Norway, Stjørdal (Værnes and Kvithamar), Melhus and Verdal (Holthe). Thirty of the isolates were collected from oat leaves (Melhus). Cultural characteristic like sporulation, colony colour, spore size and conidiophores shape were also studied on different media.

Artificial inoculation of barley in the green house (March-May 2005)

This experiment was carried out in order to establish a standard method for screening of barley breeding material in green house. Three common barley varieties were chosen, variety Gaute and Thule both strong against RLSB in the field and variety Lavrans, which is susceptible. The experiment was a completely randomised design with 3 varieties, 2 treatments (inoculated and not-inoculated) and 4 replicates. Seeds were sown in 12 cm pots in a sandy growth medium with low nutrient (March 17). Plants were fertilised (EC =1) every other day starting two weeks after sowing by an ebb and flow system. Artificial illumination (16 h/day) was with a combination of high pressure sodium lamps (32 W/m2) and NUVL (36 W/m2). Temperature was at 22 ºC during the day and 10 ºC at night. In order to induce dew,

the green house floor was sprayed with water every afternoon. Barley plants were inoculated 4 weeks after sowing (BBCH 35, April 15). The inoculum was harvested from twelve 9 cm Petri dishes of 6 different *R. collo-cygni* isolates cultured on VJA for approximately two weeks. The inoculum which was a combination of spores and mycelia was passed through two layers of cheese cloth and diluted by distilled water and 2 g/l of gelatine was added to the solution. Treated plants were sprayed to dripping point at a rate of 50 ml inoculum solution /m^2. After inoculation barley plants were kept in 100% humidity in plastic tents for three days. Each pot containing 4-5 barley plants was assessed three times starting 18 days after inoculation. A disease severity percentile was given to the whole pot.

Studying the epidemiology of **R. collo-cygni**

For three growing seasons (2000-2002) the development of RLSB on barley variety Lavrans (susceptible) was monitored on plots grown at three different sowing dates. These starting at the last week of April to mid May, each with one week intervals. Since 2000 we had two field trial series in collaboration with the local agricultural advisory office (Landbrukets forsøksringer, LFR). Details about these trials are published (Salamati, 2003). We started a new series of field trails in 2005 where we looked at the effect of diseases on barley quality as a forage crop. Therefore from 2000 at a few locations in central Norway we had detailed information on RLSB occurrence and development. Bioforsk decision support system VIPS (Varsling Innen Plante Skadegjørere) has weather stations in the most parts of central Norway where RLSB is prevalent. We looked at historical weather data in connection with the last year's final disease severity at the weather station Kvithamar. After a preliminary survey of historical weather data and the development of RLSB, we concentrated our work on precipitation and leaf wetness duration as important weather factors in connection with the final disease severity. In the first survey, leaf wetness duration (minutes, cumulative) and temperature (ºC, average) in three days intervals were correlated with final disease severity data for most of the growing season for spring barley (April-July). In the second survey in 2005 we looked at the number of days with precipitation as well.

Results and discussions

Isolation of the fungus

It is generally difficult to isolate *R. collo-cygni* by conventional isolation methods before barley plant has reached its generative phase (BBCH 45). Another problem with the isolation is the fungus sensitivity to chlorine and ethanol usually used for surface sterilisation. A major and final problem with the isolation is that we are able to isolate *R. collo-cygni* only a short period of time after the leaf is detached from barley or any other host (about three months). After this period the fungus dies (E. Sachs, http://www.bba.de/inst/a/ramularia/). Collecting young leaves at early spotting stages will increase the chance of successful isolation since they are less attacked by fast growing saprophytes.

Culture characteristics

(A) *Colour variation*

R. collo-cygni grows well but slowly on most agar media used for fungi. It is a colourful fungus. In fact the colony colour changes depending on the medium it is grown on (figure 2). The colony colour is whitish yellow (light salmon) on VJA and PCA. Gray on MA, Red on VJA + 10 mg/l maltose, pinkie white on SA, and violet on barley leaf extract agar. Undersides

of the colonies on the main medium we use (VJA) are yellow orange at the beginning (the first one to two weeks) and later reddish/violet /dark blue.
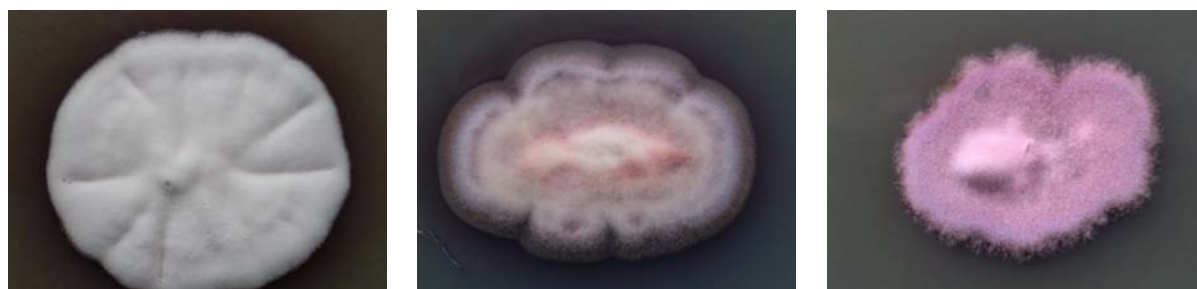
Figure 2. *R. collo-cygni* isolate T2 cultured from left to right on Vegetable Juice Agar, Straw Agar and Barley Extract Agar.

The yellow orange component (most likely rubellin B) is not soluble in the agar medium (VJA) and does not spread to the colony surroundings. The reddish/ violet/ dark blue component is mainly produced when the cultures are at least 2 weeks old (figure 3). This colour component is on the other hand soluble in the agar medium and spread around in the agar plate.
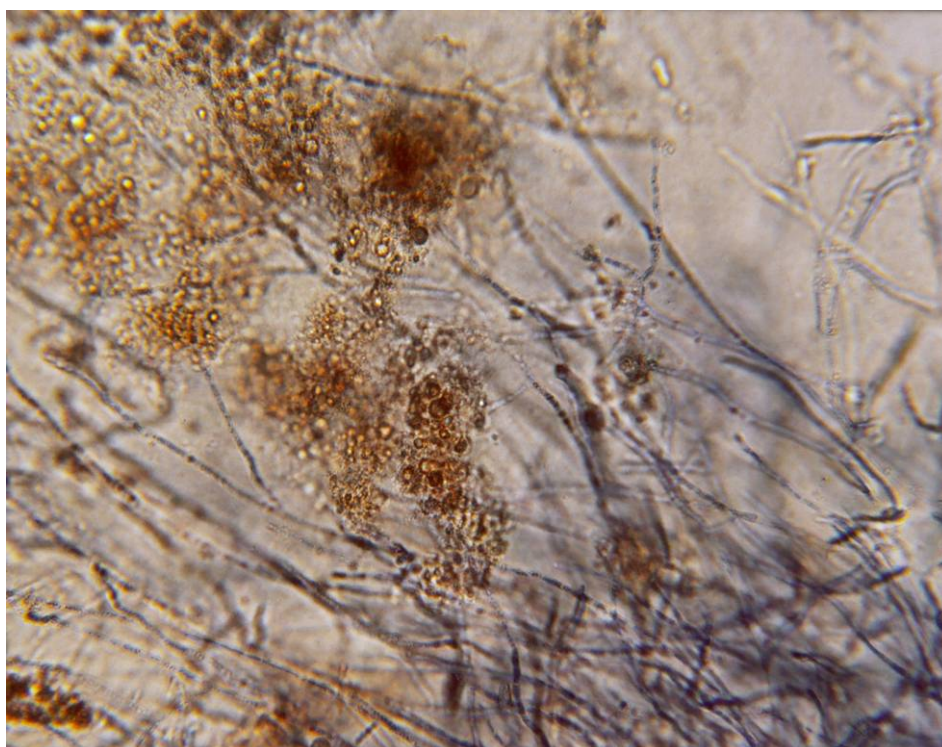
Figure 3. Rubellin production by *R. collo-cygni* hyphae. Rubellin is separated by colouring the hyphae (living tissue) with cotton blue.

The reddish/ violet/ dark blue component around colonies is produced at least in four cases: in the first case when the cultures are illuminated by excess light (cultures placed close

to sun light by the window, figure 4), the second case, when colonies compete on nutrition (figure 5a) and in reaction to an intruder, f. ex. Bacteria (figure 5b).
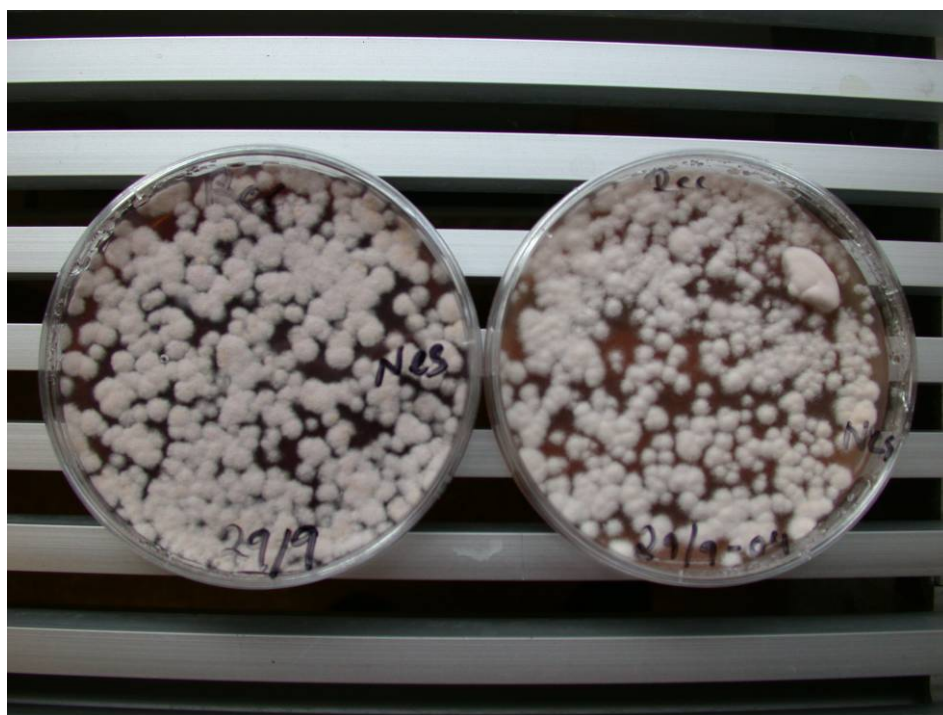
Figure 4. *R. collo-cygni* cultures produce rubellins soluble in growth medium (reddish violet colour) when illuminated by sun light (placed by the window).

a)

b)
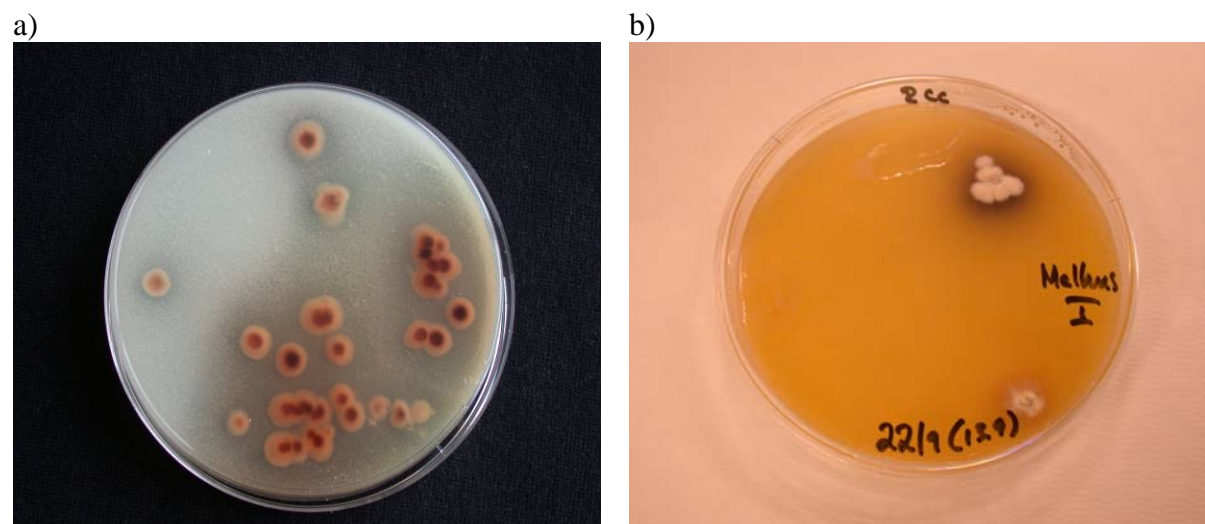
Figure 5. a) Rubellin production (undersides of colonies) enhanced in competition for nutrients between colonies of the same isolate. b) Soluble toxic metabolite produced by *R. collo-cygni* toward a bacteria colony.

There exists genetic variability in the fungus in production of soluble rubellin. Approximately 10 % of the isolates produce this soluble metabolite almost from the first day of growth (figure 6). We believe that this metabolite is rubellin D, a polar form of the rubellins which is also soluble in plant sap (Miethbauer *et al.*, 2003). What does all this

mean? We believe that rubellin D is produced when ever the fungus is threatened or in other word "stressed" in any way (signalling possibility for starvation or competition).
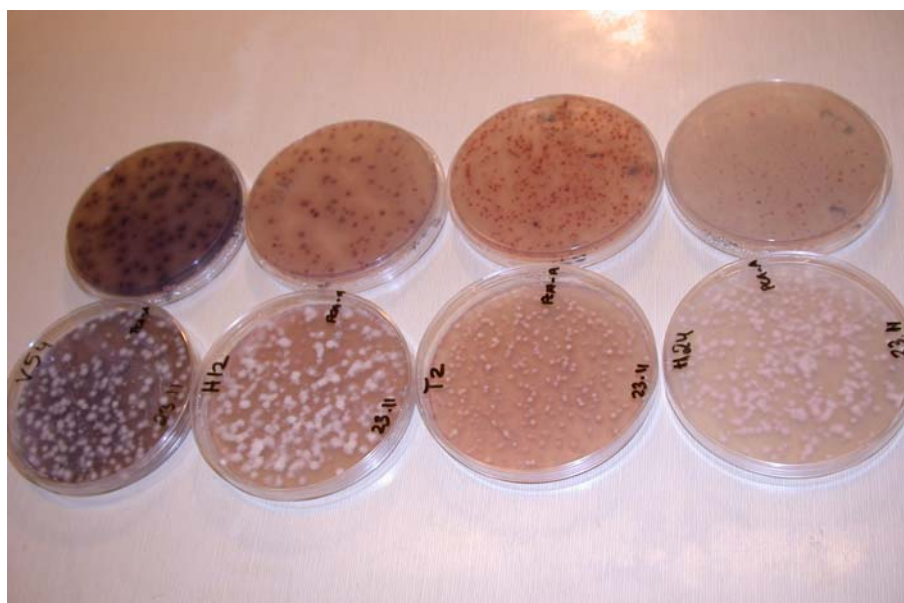
Figure 6. Variation in the production of soluble rubellin in different isolates of *R. collo-cygni* inoculated at the same date. The upper row indicates the underside of the cultures.

(B) *Spore size and the length of conidiophores*
Through the study of the cultural characteristics of *R. collo-cygni* we have found that the length of conidiophores varies depending on the growth media. The spore size is also affected by the culture media. As mentioned in the material and methods we have tested two starvation media (PCA and MEA). These media are used for inducing sporulation in fungi that do not sporulate easily in culture. Spores in these media were produced abundantly after 3 weeks (figure 7) but they were about half the size of the spores grown on VJA (figure 8). The typical conidiophores (swan necks) are also shorter on these media.
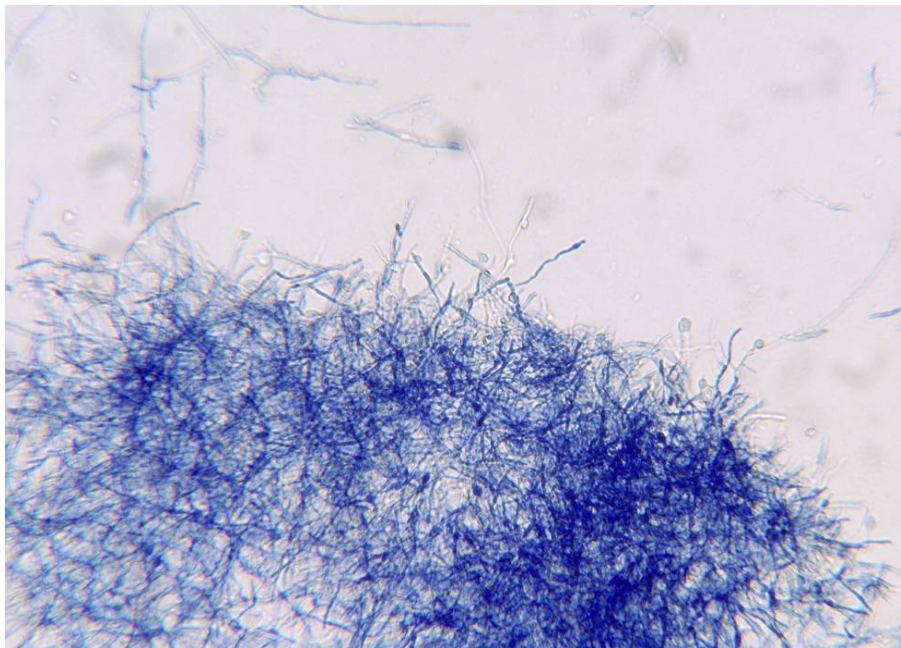
Figure 7. *Ramularia collo-cygni* sporulating on Potato Carrot Agar (prepared by adding cotton blue).
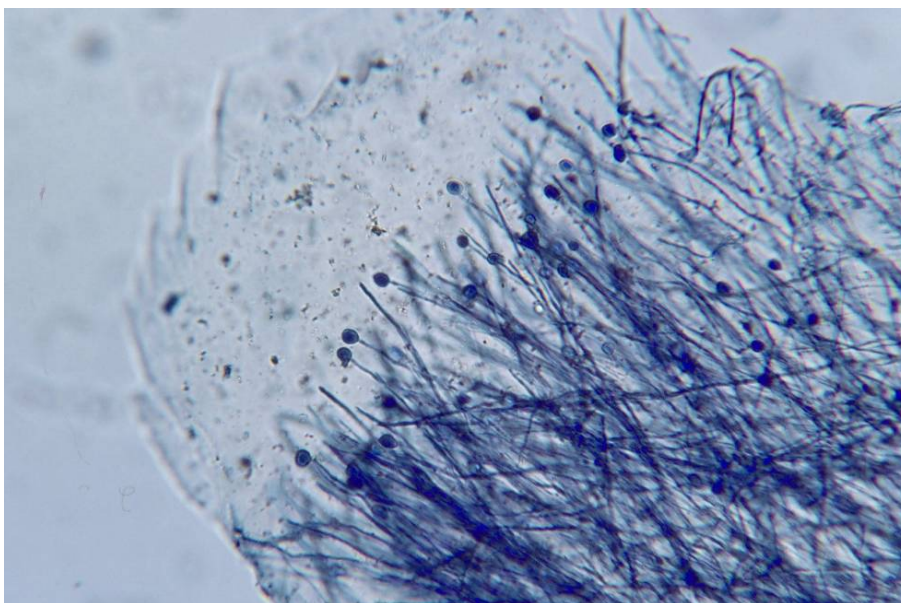
Figure 8. *Ramularia collo-cygni* sporulating on Vegetable Juice Agar (prepared by adding cotton blue).

The conidiophores are usually thinner in culture media than conidiophores sporulating on barley leaves. Sporulation is almost none existing in cultures grown in the dark and the old cultures (over 4 weeks old) would not sporulate willingly. The majority of the isolates sporulate after 6 to 7 days on VJA under conditions mentioned in growth cabinet. At temperatures over 20 ºC spores germinate in a few minutes (figure 9) and at about one hour at room temperature (22-25), no visible spore is found on the plates.

Figure 9. *Ramularia collo-cygni* spores germinate shortly after being at temperatures over 20 ℃.

(C) *Asteromella* stage of *R. collo-cygni*

Looking at a few old cultures of the fungus (6 months old, kept at 4 ℃) we recognised pycnidia-like organs at the edges of the colonies. These were of various sizes, a few visible by the naked eye. Old isolates generally produce swollen hyphae toward the edges of the colonies and remaining food supply (figure 10). These organs later develop to a spermogonial stage which is called *Asteromella*.
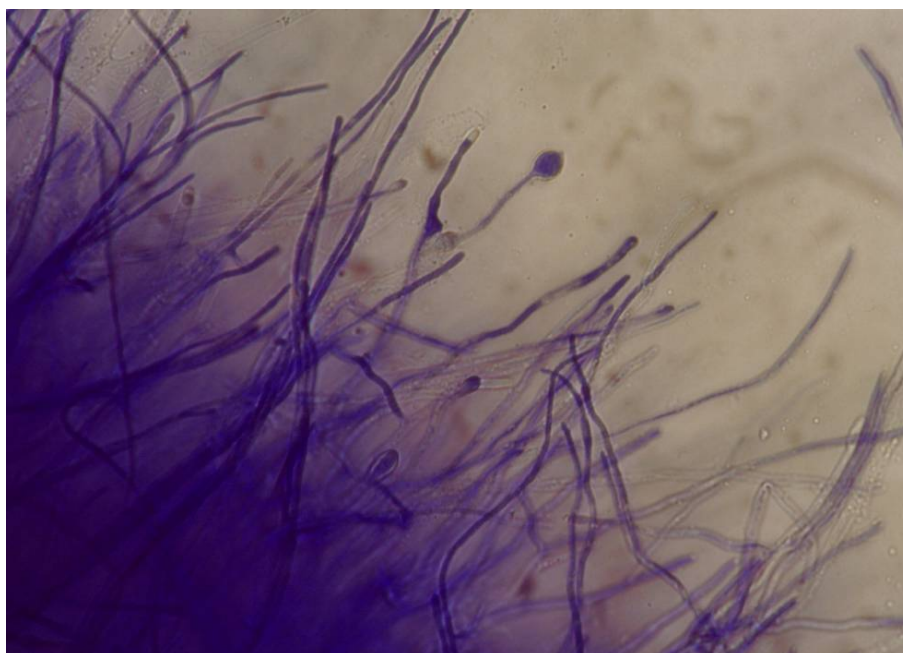
Figure 10. In the old cultures, *R. collo-cygni* produces swollen hyphae toward the edges of the colonies (prepared by adding cotton blue).

We added a few autoclaved pieces of barley straw to some three weeks old cultures of *R. collo-cygni* in order to see if it produces similar organs on its original host. Indeed after one week the fungus produced mainly these spermagonia both over and inside the barley straw (figure 11). Six of our isolates were sent to Centraalbureau voor Schimmelcultures (CBS), the Netherlands. They confirmed the fungus identity by molecular methods and its new structure (Prof. Perdo Crous, pers. comm.).

Asteromella stage of *R. collo-cygni* has not been reported from *in vitro* cultures earlier. Braun (2002) reported about these structures on barley leaves with RLSB symptoms from a publication in Argentina. Now with documenting the existence of the *Asteromella* stage of *R. collo-cygni*, its telomorph if existing with no doubts would be *Mycosphaerella* as suggested by Braun (2004), Miethbauer *et al.* (2003) and Heiser *et al.* (2004). In one plate (isolate M74), we found a spermogonium in a position very similar to conjugation (figure 12). We were not able to follow the process since the fungus was killed by the preparation method.
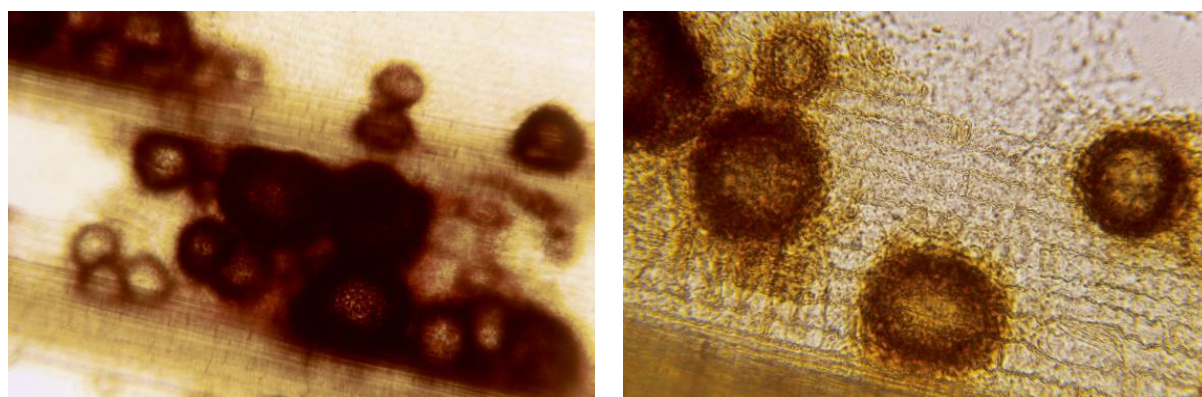
Figure 11. Spermogonia (*Asteromella* stage) of *Ramularia collo-cygni* produced on autoclaved barley straw in vitro.
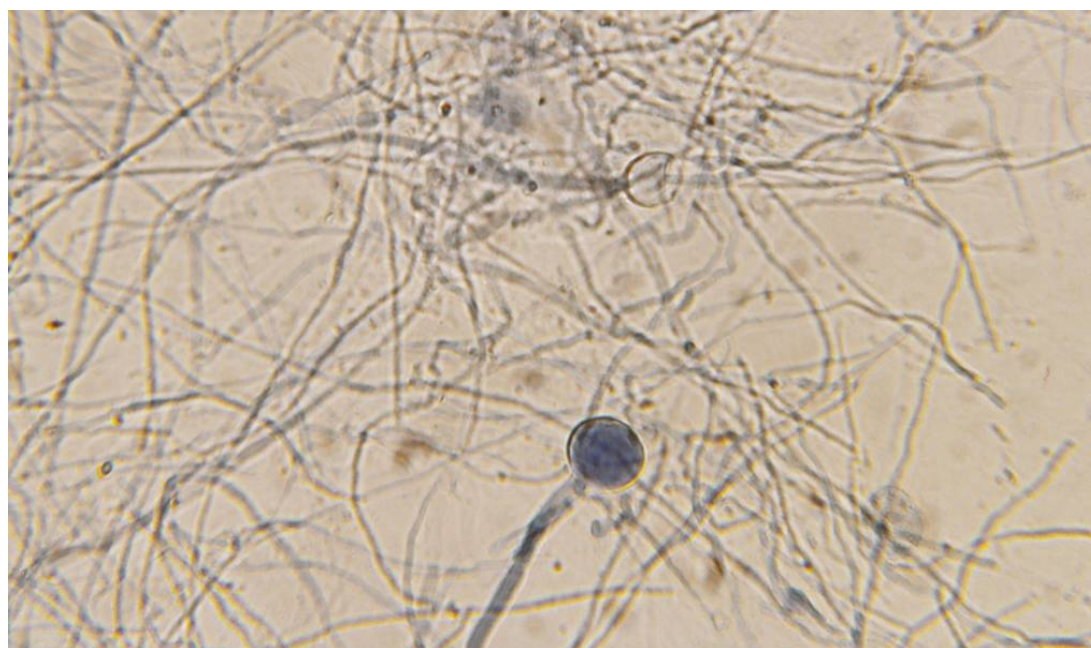
Figure 12. A spermogonium of *Ramularia collo-cygni* with a receptive hyphae (prepared by adding cotton blue).

Artificial inoculation of barley in the green house (March-May 2005)
The inoculated barley varieties were assessed three times. Typical RLSB symptoms started approximately two weeks after inoculation mainly on the susceptible variety Lavrans. The maximum disease severity at BBCH 50 was 15%, also the same at BBCH 55. At BBCH 70 it reached 40% (figure 13).

Figure 13. Variety Lavrans artificially inoculated by a combination of six isolates of *Ramularia collo-cygni*.

Figure 14. Barley leaves artificially inoculated in the green house (BBCH 75).

Epidemiology of **R. collo-cygni**

Our study of the weather parameters leaf wetness duration and temperature (Salamati, 2003) showed that the most important factor for a severe infection of RLSB in central Norway is long leaf wetness durations only the first 2 weeks of June. In 2005 we looked in more detail at this period. Looking at the data for six years in this period showed a strong correlation between the leaf wetness duration as well as the number of days with precipitation and the final RLSB severity for the coming growing season (figure 15). Our conclusion after these two surveys is that barley is infected and RLSB is established in this period (the first 10 days of June). In central Norway, the barley plant is then at BBCH 15-30 depending on the sowing time. Infection can happen later in the growing season but would not cause considerable economic loss. The disease is seldom detected before the start of the generative phase of barley.
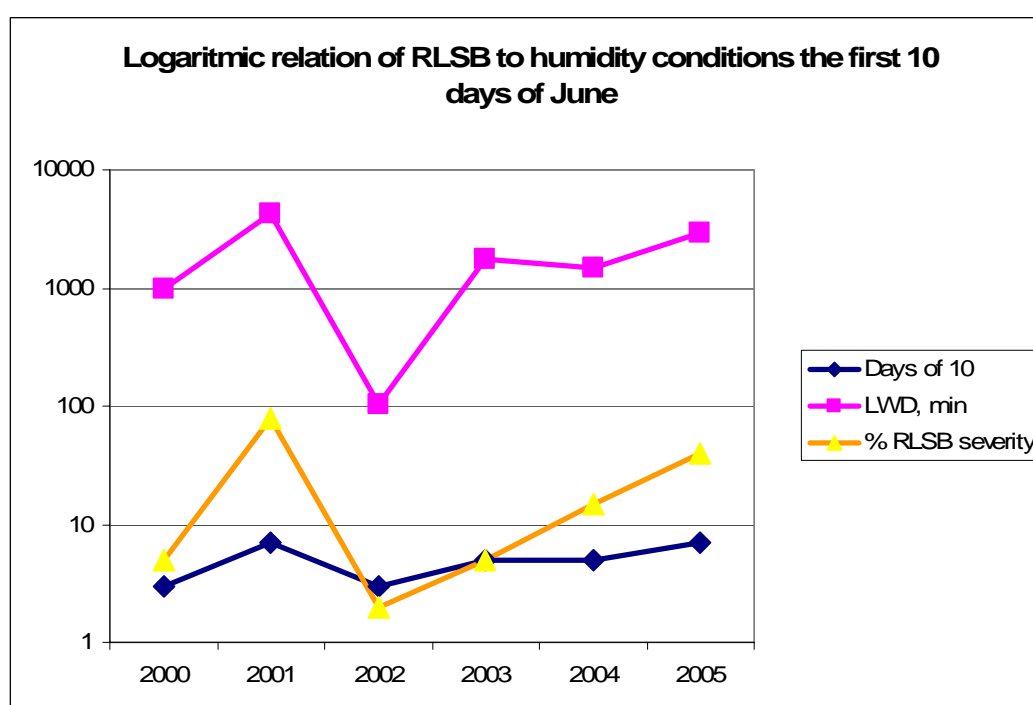
Figure 15. Epidemics of Ramularia Leaf Spot on spring barley in Norway are strongly correlated with humid conditions the first 10 days of June (Leaf Wetness Duration, LWD and number of days with precipitation, weather station Kvithamar).

In fact *R. collo-cygni* starts sneakingly in the barley fields early in the growing season. Few lower leaves are attacked and symptoms are rather different from the symptoms at generative phase of barley. They appear as few relatively large spots similar to the letter " H " with up to 5 mm length and 3 mm wide (figure 16). Spots at this stage are quite similar to the spots we find at maturity on our "tolerant" varieties f. ex. Gaute in the field. After the senescence of the lower leaves, the barley plant is asymptomatic and systemic in the plant until the generative phase starts. Asymptomatic infection of barley has been confirmed by PCR methods (Havis *et al.*, 2004). We also showed it in our artificial inoculation test mentioned in this paper.

R. collo-cygni can sporulate abundantly, spores are small, about 1/10 of the spores of *Blumeria graminis* which has airborne spores. In addition, *R. collo-cygni* spores are equipped with warts and can easily attach to any air particle. Based on the work carried out by

Minihofer (2003) calculated a mass of approximately 3×10^{12} spores /ha from a severely attacked barley field. Huss *et al.* (2005) reported that *R. collo-cygni* is not dependent on any physiological pause and actually can sporulate under the snow. The fungus can have several sources of inoculum some of which are shown in the following figure (17).

Figure 16. Early symptoms of Ramularia Leaf spot of barley with few large spots on lower leaves.
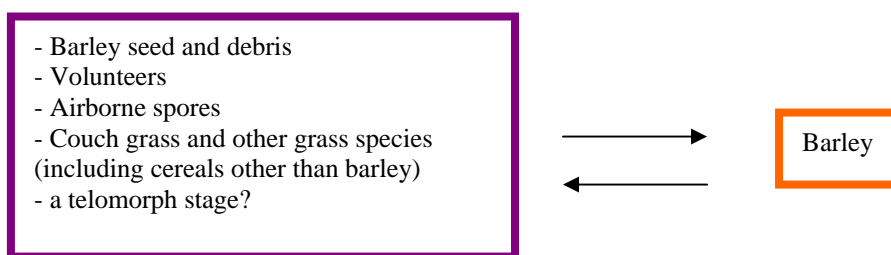
Figure 17. Possible sources of inoculum for Ramularia Leaf Spot of Barley.

We do not grow winter barley in Norway. In the countries where winter barley is cultivated, the most convenient source of inoculum for spring barley is winter barley. The humid weather during autumn gives perfect conditions for an early infection and later the large areas cultivated by winter barley would provide ready inoculum for the spring barley infection. In Norway we found *R. collo-cygni* on oat and couch grass for the first time in 2005. We believe the perennial couch grass is one of the most important sources of inoculum in central Norway. We have south eastern winds coming from Scotland. Wind borne inoculum can also be an important source for our spring barley fields. When winter barley is harvested in northern Europe, large masses of conidia are flown to the air. Most European pathotypes of *B. graminis* have been found in Norway. There is no reason for *R. collo-cygni* conidia not reaching Norway through the same path as *B. graminis*. Harvey (2002) reported an infection rate in barley seeds as high as 10%. Seed infection can also be an important factor for epidemics of RLSB as well. In addition to an important source of epidemics of RLSB, seed infection can introduce the fungus and its novel genotypes to new areas.

Why *R. collo-cygni* *started being a barley pathogen in early 80s?*

The fungus was first reported in 1893 by Carava. It was also reported by Jørstad in Norway in 1930. He concluded that *R. collo-cygni* was most probably a saprophyte. In Europe the fungus was first reported as a possible causal agent of typical speckled barley 57 years later (Huss *et al*, 1987). Several features of *R. collo-cygni* are similar to fungal endophytes. Table 1 shows some of its characteristics in comparison with a major well known pathogen of barley *Rhynchosporium secalis*.

Fungal endophytes (family *Clavicipitaceae, Ascomycetes*) infect several grass species (Clay, 1989). They produce physiological active alkaloids in the tissue of their host. Infection makes grasses toxic to domestic mammals and increase resistance to insect herbivores.

The association between *R. collo-cygni* and barley plant seems sophisticated, old and complicated. The fungus has several features of endophyte as shown in table 1. Rubellin production can be one of the reasons that plant permits infection and colonisation of *R. collo-cygni* in the first place, even though these toxins can eventually cause plant premature ripening. Rubellins as shown by Heiser *et al.* (2003, 2004) are general toxins. They might confer protection against insects or other intruders. Miethbauer *et al.* (2003) found that the main rubellin type in barley leaves is rubellin B which is not soluble *in planta*. Heiser *et al* (2004) implied that *R. collo-cygni* produces protective compounds similar to pyridoxine (vitamin B6) in order to protect itself against rubellins (the barley plant is protected as well while it is healthy and young). These protective compounds (e. g. pyridoxine) are synthesised by nutrients provided by the host. This might be the reason that *R. collo-cygni* dies in a few weeks after leaf detachment and its isolation would not be possible after this period (the nutrient source does not exists any more). In the field, old infected leaves are decomposed by

soil bacteria and fungi and *R. collo-cygni* can live saprophytically without being killed by its own toxins.

Table 1. Comparison of *Ramularia collo-cygni* with *Rhynchosporium secalis* in relation to typical fungal endophytic characteristics.

Endophytes:	R. collo-cygni	R. secalis
Grow intercellularly in plant tissue	√[a]	-[b]
Can infect several plant species	√	-
Are often involved in plant litter decomposition	√[c]	-
Usually do not provoke resistance mechanisms in plants	√	-
Can be asymptomatic for a long time in plant tissue	√	-
Sporulation is seen after senescence of plant tissue	√	-
often contain toxic alkaloids	√	-

[a] Sutton & Waller, 1988; [b] Xi *et al.* ,2000; [c] Salamati, 2003.

R. collo-cygni has also a close relative, *Asteromella* sp. being one of the most common endophytes on Emory oak leaves in the USA (Faeth & Hammon, 1997).

Concluding remarks

Hypothesis on **R. collo-cygni** *- Barley pathosystem*
Based on the studies on the production of rubellins by *R. collo-cygni* (Heiser *et al.* 2003, 2004 and Miethbauer *et al.* 2003) and our studies on the colour components variability *in vitro* we like to hypothesise that *R. collo-cygni* is an endophyte, but has recently evolved to a latent pathogen. Infection and colonisation is inevitable in locations prone to RLSB, when humid weather conditions (fog, dew, high humidity) prolong early in the growing season. We demonstrated that the fungus produces soluble rubellin in stressful conditions. Breeding studies in Norway and other countries have shown that the disease is usually more serious on 2-rowed barley varieties (narrow leaves that senescence early) and the varieties with resistance genes against other barley pathogens. We propose that introduction of resistance varieties that are easily stressed by biotic and abiotic factors has caused selection of the *R. collo-cygni* genotypes that are easily stressed (produce more rubellin or soluble rubellin with the slightest stress).

A possible scenario would be the following; when the host is stressed, *R. collo-cygni* would also be stressed (sensing possibility for starvation) and produces more rubellin. In order to leave a "dying" host full of rubellin, the fungus sporulates. Open stomata in humid conditions are the easiest path to leave the plant. The fungus literally senses the light from open stomata and more rubellin is produced that would kill the nearest tissue stopping the plant from closing its stomata. The whole process happens eventually in all other infected barley varieties, but when the plant has reached senescence.

The strongest barley varieties we have lack any known resistance gene; they are six-rowed and have usually broad leaves with a green healthy appearance.

R.collo-cygni usually loses the competition with the typical barley pathogens like *Rhynchosporium secalis*, *Pyrenophora teres* and *Blumeria graminis*. Improved disease management against these pathogens in the last decades has reduced its competitors and given *R. collo-cygni* a better chance for establishment in the barley fields.

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Scanning electron microscopic investigations on leaves of barley and maize infected with *Ramularia collo-cygni*

Edith Stabentheiner[1]), Tobis Minihofer[1]), Herbert Huss[2])
[1]) *Institute of Plant Sciences, University of Graz, Schubertstraße 51, 8010 Graz, Austria;*
[2])*Institute of Organic Farming, HBLFA Raumberg-Gumpenstein, Field Trial Station Lambach-Stadt-Paura, Gmundnerstraße 9, 4651 Stadl-Paura, Austria*

Abstract: *Ramularia collo-cygni* B. Sutton & Waller was investigated using scanning electron microscopy on leaf samples of barley and maize sampled in the summer and the winter season. The host is invaded via the stomata. The conidiophores emerge through the stomata but are also capable of breaking through the epidermis of heavily infected leaves. Caespituli are concentrated in patches, but cover the surface completely on heavily infected chlorotic and necrotic leaves, with the highest density always on the lower surface. *R. collo-cygni* is able to infect and sporulate on necrotic, chlorotic and green leaves in summer and winter conditions with low temperatures seeming to have no effect on either its morphology or life cycle.

Investigating the life cycle of *Ramularia collo-cygni* using a PCR based diagnostic

Neil Havis and Simon Oxley

Scottish Agricultural College, West Mains Road, Edinburgh, Scotland, EH9 3JG, UK.

Abstract: The life cycle of the barley disease, *Ramularia collo-cygni* was studied using a recently developed PCR based diagnostic test. The appearance of the pathogen was monitored in field trials over a number of seasons. Controlled environment experiments focused on a potential seed-borne stage for the fungus. Results indicated that the fungus can be detected before the appearance of visible symptoms but the earliest detection date varies between season and variety. In addition the pathogen is widespread at the end of the growing season in harvested grain samples and can be transmitted to developing plants from infected seed stock. Examination of infected seedlings did not reveal the presence of spores but fungal structures were found within the leaf. The implications of potential seed-borne stage in the pathogen life cycle are discussed.

Key words: *Ramularia collo-cygni*, detection, pcr, transmisson, life cycle

Introduction

Ramularia collo-cygni is an increasingly important late season pathogen of barley (*Hordeum vulgare*) both in Europe and the United Kingdom (Sachs *et al.*, 1998; Pinnschmidt & Hovmøller, 2003; Oxley & Havis, 2004). Disease symptoms appear on foliage after the emergence of the ear and contribute to premature loss of green leaf area (Havis *et al.*, 2004). The impact of this pathogen on yield can be as high as 0.36 t/ha in susceptible years (Oxley & Havis, 2004). The development of a molecular based diagnostic test for *R. collo-cygni* has allowed more detailed studies of the ecology, aetiology and epidemiology of this poorly studied pathogen (Havis *et al.*, 2006). Visual crop assessments and diagnostic analysis has indicated that the pathogen moves up the plant during the growing season and also infects awns and ears (Oxley & Havis, 2004). In this paper we describe some of the experiments carried out to elucidate the life cycle of *R. collo-cygni*.

Material and methods

R. collo-cygni *detection*
A series of field trials have been carried out in Scotland to determine the most cost effective fungicide programmes to control *R. collo-cygni*. Leaf and grain samples have been collected from these trials throughout the growing season and tested for the presence of the pathogen using the method described in Havis *et al.* (2006).

Monitoring **R. collo-cygni** *in seed*
Seed samples from untreated plots from SAC trials were collected for testing for the presence of *R. collo-cygni*. Samples of 100 seeds were ground in a Kenwood food processor (Kenwood, UK) for 5 minutes until they reached the consistency of a fine powder prior to DNA extraction (Lee *et al.*, 2001). DNA was then extracted using the Sigma REDExtract N-Amp ™ kit and a nested PCR reaction carried out as described in Havis *et al.*, 2006. In 2005 a selection of spring barely seed samples from around Scotland were supplied by Dr. Valerie Cockerell (Official Seed testing station for Scotland) and tested using the described method for the presence of *R collo-cygni*.

Transmission of **R. collo-cygni** *to developing plants*
An experiment was undertaken to study the potential transmission of *R. collo-cygni* from seed to emerging and developing plants. Seeds were sown into Levington F2 compost and grown in a controlled environment chamber. Nine seeds were sown in each 18 cm pot. After emergence the plants were thinned to six per pot. Plants were grown at 18 °C with a 16-hour daylength. When three true leaves had unfurled, leaf samples were harvested and frozen at -20°C prior to testing. Two leaf discs were taken at random from the harvested leaves using a hole punch for DNA extraction and each sample was tested three times.

Microscopic examination of plant material
Leaf material from seed samples, which gave a positive PCR result, was examined microscopically for the presence of fungal spores. Green leaves were cleared of chlorophyll using a method modified from Ryan and Clare, 1974 (J Fontaine, pers. comm.). Leaves were placed in a petri dish on filter paper soaked with 1:1 (v/v) solution of glacial acetic acid and absolute ethanol. The plates were exposed to daylight for 4 days and then the leaves removed and added to petri dishes containing filter paper soaked in distilled water for a further 3 days. The leaves were then stained with Trypan/blue lactophenol for varying time periods. Leaves were then mounted on a microscope slide in clear lactophenol under a coverslip, which was sealed with nail varnish.

Results and discussion

R. collo-cygni *detection*
Results from testing of samples from five years of field trials indicate that *Ramularia* can be detected prior to the appearance of visual symptoms in the crop.

In this trial all of the untreated varieties gave a positive result for the presence of *R. collo-cygni* 60 days after sowing. This indicates that varieties do not differ in their ability to resist initial colonisation by the fungus. However the severity of disease symptoms when expressed as the area under the disease progress curves was shown to differ widely between varieties. The highest figure was recorded by the variety Optic (249, Figure 1) and the lowest by the new variety Poker (50, Figure 1). Disease progress curves and detection dates vary from season to season and also between sites.

Results indicate that the PCR based diagnostic was able to detect the presence of the fungus prior to the appearance of symptoms. The maximum interval between detection and appearance was 28 days and the minimum was 0 days. Longer intervals appeared to coincide with higher disease years.
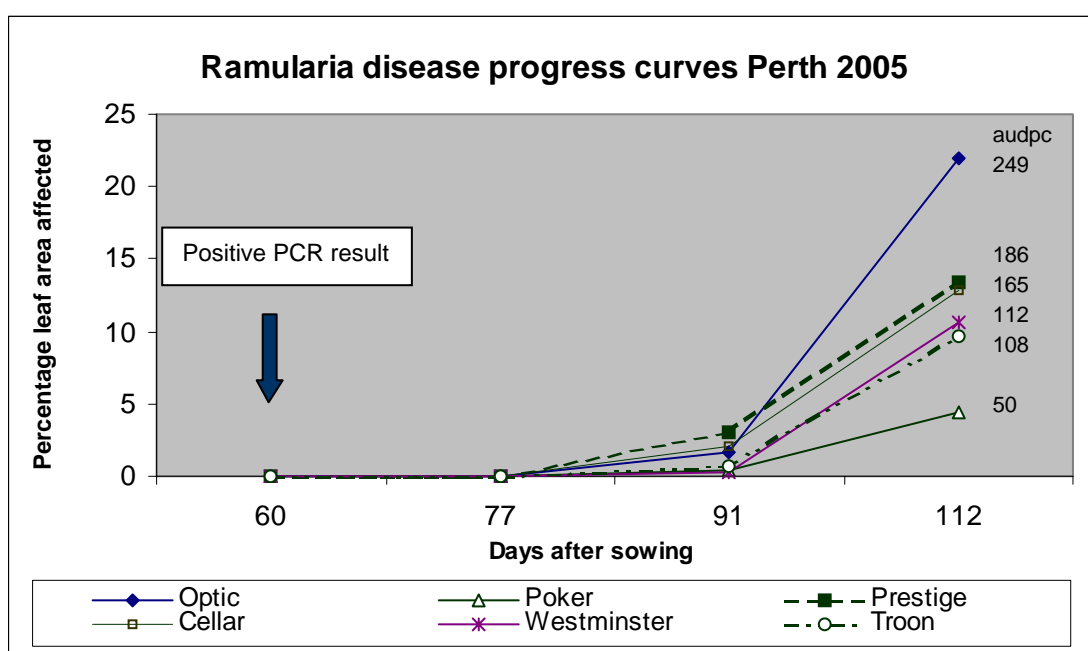
Figure 1. Development of *R. collo-cygni* symptoms at a spring barley trial site in 2005.

Table 1. Use of PCR diagnostic to detect pre-symptomatic infection of barley in field trials. Figures are the means from a minimum of two field trials per year.

Year	W Barley		Spring Barley	
	Days between detection and visual expression	**Maximum leaf area infection (untreated)**	**Days between detection and visual expression**	**Maximum leaf area infection (untreated)**
2000	-	-	21	37%
2001	-	-	0	6%
2002	-	6%	3	28%
2003	-	9%	12	14%
2004	27	5%	18	28%
2005	28	23%	28	26%

Monitoring **R. collo-cygni** *in seed*

The life cycle of *Ramularia collo-cygni* is poorly understood. Preliminary schemes have the fungus overwintering on winter crops, volunteers and secondary hosts prior to transferring to spring crops (Sachs, 2002). The infected spring crops then act as a source of inoculum later in the growing season for the winter crops. Anecdotal evidence from Austria indicated that there could be a potential seed-borne stage in the life cycle (H. Huss pers. comm.). Previous studies have indicated that the fungus moves up the crop during the growing season (Salamati, 2002; Oxley & Havis, 2004) but the initial infection source has not been clearly identified. A number of pathogens of barley are known to be seed borne e.g. *Rhynchosporium secalis, Pyrenophora teres, Pseudoseptoria stomaticola* (Murray *et al.*, 1998). Our results demonstrate that the proliferation of *R. collo-cygni* spores late in the growing season has lead

to the infection of all of the barley seed samples tested. *Ramularia* spores have been detected on leaves of oak trees late in the growing season in Germany (Heuser & Zimmer, 2001).

Table 2. Seed samples which gave a positive result for *R.collo-cygni* with PCR diagnostic

A

Crop	Region	Year	Varieties
S Barley	Aberdeenshire	1999	Henni, Century, Optic, Cooper, Delibes, Chariot, Prisma, Newgrange

B

Crop	Variety	Year	Region
W Barley	Sumo	2004	Midlothian
W Barley	Haka	2005	Perthshire
S Barley	Cocktail	2004	Clackmannanshire, West Lothian
S Barley	Chalice	2004	Clackmannanshire, Midlothian, Fife
S Barley	Cellar	2004	Perthshire
S Barley	Decanter	2004	West Lothian, Perthshire, Orkney, Aberdeenshire
S Barley	Golf	2004	Aberdeenshire
S Barley	Golden Promise	2004	Orkney, Aberdeenshire
S Barley	Hart	2004	Aberdeenshire
S Barley	Maresi	2004	Fife, Falkirk
S Barley	Optic	2004	Fife, Angus, West Lothian, East Lothian, Roxburghshire, Perthshire, Kelso
S Barley	Prestige	2004	Kincardineshire
S Barley	Pewter	2004	Caithness
S Barley	Rebecca	2004	Fife
S Barley	Riviera	2004	Aberdeenshire, Caithness, Lanarkshire, Dumfriesshire, Banffshire, Fife
S Barley	Static	2004	Aberdeenshire
S Barley	Tyne	2004	Caithness, Orkney, Aberdeenshire
S Barley	Troon	2004	Rossshire, Fife, Clackmannanshire, Aberdeenshire

Transmission of **R. collo-cygni** *to developing plants*

The testing of barley plants grown from this infected seed demonstrates that the fungus is being transferred from seed to plant. Microscopic examination of the leaf revealed no spores present on the leaf surface. The presence of fungal hyphae in the sub stomatal cavity points to the fungus having successfully invaded the leaf tissue at an earlier date. Previous SEM work in the laboratory has shown the presence of a complex network of fungal hyphae within the leaves of *R. collo-cygni* infected plants (Havis *et al.*, unpublished results).

The application of Raxil S® (tebuconazole and triazoxide) seed dressing to winter barley seed did not appear to eliminate the presence of *R. collo-cygni* or hinder the transfer of the pathogen to the developing plant. Diagnostic techniques have been used previously to determine the location of fungal material in seed e.g. mycelia from the loose smut fungus, *Ustilago nuda* have been found within the non-embryo half of barley seed (Eibel *et al.*, 2005). The results from our laboratory appear to indicate that *R collo-cygni* may also be situated within the seed. However, further work is required to prove this theory.

Table 3. Seedlings tested for the presence of *R. collo-cygni*

Crop	Variety	Leaf One	Leaf Two	Leaf Three
S Barley	Delibes	+	+	+
S Barley	Century	+	+	+
S Barley	Henni	+	+	+
S Barley	Prisma	+	+	+
S Barley	Newgrange	+	+	+
S Barley	Chariot	+	+	+
S Barley	Cooper	+	+	+
S Barley	Optic	+	+	+
W Barley	Sumo (Raxil treated)	+	+	+
W Barley	Sumo (Untreated)	+	+	+

+ = Ramularia DNA detected

The results indicate that the *Ramularia* DNA is present on the emerging leaves of plants sown from infected seed. The presence of a seed dressing on the winter barley has not stopped the movement of the fungus from seed to plant. This contrast sharply with the case of *Rhynchosporium secalis*. This pathogen was also detected on the seed tested but the Raxil S® treatment lead to complete degradation of the fungal DNA.

Microscopic examination of plant material
Microscopic examination of cleared leaf tissue did not yield any spores on the surface of the leaf. However longer staining of the leaf segments indicated the presence of hyphal structures in the sub-stomatal cavity.

The presence of a significant seed-borne stage for this pathogen will have implications for all barley growers. *R. collo-cygni* may be present in a majority of crops prior to symptom expression. Protectant late season fungicide applications still represent the most effective way of checking the development of the fungus and also its movement onto emerging ears. Previous work has shown that untreated ears can become infected by *Ramularia* as early GS 61 (Oxley & Havis, 2004). Furthe*r* work is also required to examine the effectiveness of seed dressings in controlling the transmission of the fungus.

The presence of a seed-borne stage in the fungal life cycle would help explain the transfer of the fungus from season to season.

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Ramularia leaf spot in barley. A new disease in Sweden?

Annika Djurle [1] and Morten Rasmussen [2]
[1] *Department of Forest Mycology and Pathology, Swedish University of Agricultural Sciences, Box 7026, SE-750 07 Uppsala, Sweden.* [2] *Swalöf Weibull AB, BA Cereals, Cereal Breeding Department Landskrona, Disease Resistance Lab, SE-268 81 Svalöv, Sweden*

Abstract: The barley pathogen *Ramularia collo-cygni* has recieved increasing attention in Europe during the last years. It was found in Sweden on two occations in 2002 (M. Rasmussen and S. Salamati, pers. comm.). During 2005 spring barley fields in Sweden were surveyed for *R. collo-cygni* and samples with possible symptoms were sent in for diagnosis. The fungus was present in 13 out of 42 fields and in 3 out of 7 samples. The presence of the pathogen in different geographical areas was thereby confirmed. It is assumed that occurrence of Ramularia leaf spot often has been hidden behind other diseases and the diagnosis of physiological leaf spots (Twengström *et al.*, 2004). For 2006 field experiments addressing questions about cultivar susceptibility, yield loss and control are planned in a field where last year's barley crop was diseased by *R. collo-cygni.*
Disease scorings from these trials will be compared to similar datasets from locations in Scotland, Norway and Germany. A preliminary study on Nordic spring barley material from NGB indicates differences in resistance reactions between locations. Knowledge on pathogen population structure and dynamics as well as on pathogen virulence and plant genetic resistance interactions is still too limited for any substantial interpretation of these differences. This work is in part financed by the Swedish Farmer's Foundation for Agricultural Research.

Key words: *R. collo-cygni,* survey

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Monitoring the epidemics of *Ramularia collo-cygni*: Comparison of varieties, sites, years and methods

Michael Heß, Roland Habeker, Hans Hausladen, Ingrid Heiser
Lehrstuhl für Phytopathologie, Wissenschaftszentrum Weihenstephan der Technischen Universität München, Am Hochanger 2a, D-85350 Freising

Abstract: Although recent research established *Ramularia collo-cygni* as an important cause of leaf spotting on barley, epidemics remain mostly unsure and difficult to investigate.

The main method for quantifying the pathogen is the diagnosis of sporulation with the swanneck shaped conidiophores on the leaf surface with a microscope. Typically for these observations none or hardly any sporulation is found on the host plant in spring until mid May. About 2 weeks after flowering small necrotic lesions appear on the upper leaves followed by rapid breakdown of leaf tissue and heavy sporulation of *Ramularia collo-cygni* on the necrotic leaves.

Sporulation on the different leaf levels is compared with the occurrence and progress of the leaf spots. The results from trials from the past 5 years show influence from years, sites, variety and growth stage of the host plant on the epidemics of *Ramularia collo-cygni*. This method to observe the sporulation remains laborious and not sensitive enough for the onset and rapidity of the epidemics, since sporulation follows necrosis.

Additional observations on the epidemics of *Ramularia collo-cygni* were made collecting spores. The number of spores is compared to assessed progress of necrotic leaf area and the area with sporulation of *Ramularia collo-cygni* found under the microscope.

Using molecular methods PCR testing showed the presence of *Ramularia collo-cygni* DNA about 1 week prior to first spotting.

The results obtained with different methods will be discussed and conclusions for the epidemics of *Ramularia collo-cygni* will be drawn.

Key words : *Ramularia collo-cygni,* epidemiology, leaf spotting, PCR, sporulation

Introduction

Heavy leaf spotting followed by sudden breakdown of the crops is becoming a major threat to yield expectations of winter and spring barley in Germany. About 2 weeks after flowering small necrotic lesions appear on the upper leaves followed by rapid breakdown of leaf tissue and heavy sporulation of *Ramularia collo-cygni* on the necrotic leaves. Still epidemics remain mostly unpredictable and difficult to investigate. The knowledge of the epidemiology of *Ramularia collo-cygni* is fundamental for appropiate control measures.

The main method for quantifying the pathogen is the diagnosis of sporulation with the swanneck shaped conidiophores on the leaf surface with a microscope. Typically there is none or hardly any sporulation observed on the host plant in spring until mid May. It is difficult to relate the sporulation to the progress of the necrosis and breakdown of the crop. The comparison with other, more sensitive, methods is needed for a better understanding of the epidemiology of *Ramularia collo-cygni*.

In addition to the occurrence of *Ramularia collo-cygni* the variety, site and the year of the observation showed great impact on the disease. The influence of these mostly abiotic factors have to be taken into account regarding *Ramularia collo-cygni* epidemics, since the phenomenon of this late leaf blight in barley shows strong interaction with physiological aspects.

Material and methods

Sites and Years

Field trials were carried out at different sites in the area of Weihenstephan since 2001. The altitude of the sites was between 470 m and 510 m with similar climate (precipitation 800 mm, average temperature 7-8 °C). The sites in Weihenstephan and Zurnhausen had heavy, loamy soil. At the site Lohhof the soil is light with high level of organic matter and weak water holding capacity. Although there are small regional and climatic differences between the sites, great differences in *Ramularia collo-cygni* epidemics were observed.

For comparison of epidemics the following data were recorded: Date of flag leaf unrolled (BBCH 39), first spots on the upper leaves (flag or F-1), breakdown (100% necrosis) of the crop, first sporulation of *Ramularia collo-cygni,* PCR positive (site, F-1).

Varieties

The trials included the 2 row winter barley varieties Duet, Tafeno, Carrero, Camera, Jessika, the 6 row winter barley variety Merlot and the summer barley variety Auriga. All varieties except for Merlot showed high susceptibility to leaf spotting.

Methods

The main method for quantifying the pathogen is the diagnosis of sporulation with the swanneck shaped conidiophores on the leaf surface with a microscope. Sporulation on the different leaf levels is compared with the occurrence and progress of leaf spots.

Additional observations were made collecting spores. Spores were washed off the surface from 40 sampled leaves per leaf level and counted in a Thoma counting chamber as spores/ml. The number of spores is compared to the assessed progress of necrotic leaf area and the area with sporulation of *Ramularia collo-cygni* found under the microscope.

PCR diagnosis of the 2004 samples was carried out by Neil Havis, SAC Edinburgh. The 2005 PCR investigations were carried out in Weihenstephan, following the method from the SAC (Havis *et al*., 2006).

Results and discussion

Variety

In the 2005 winter barley trial in Weihenstephan the 2 row winter barley varieties Duet, Jessika, Camera and Carrero were compared to the 6 row winter barley Merlot. The variety Merlot shows good resistance to the typical leaf spotting. It is evident, that the variety Duet showed less severe symptoms at the time of assessment compared with the other 2 row varieties, although Duet is generally regarded as an highly susceptible variety and showed heavy symptoms later in the growing season (figure 2). The varieties Jessika, Camera and Carrero with the later growth stage (BBCH 85) showed more severe symptoms at the time of

assessment. These results indicate that plant maturity has to be taken into account when comparing varieties for resistance to leaf spotting.
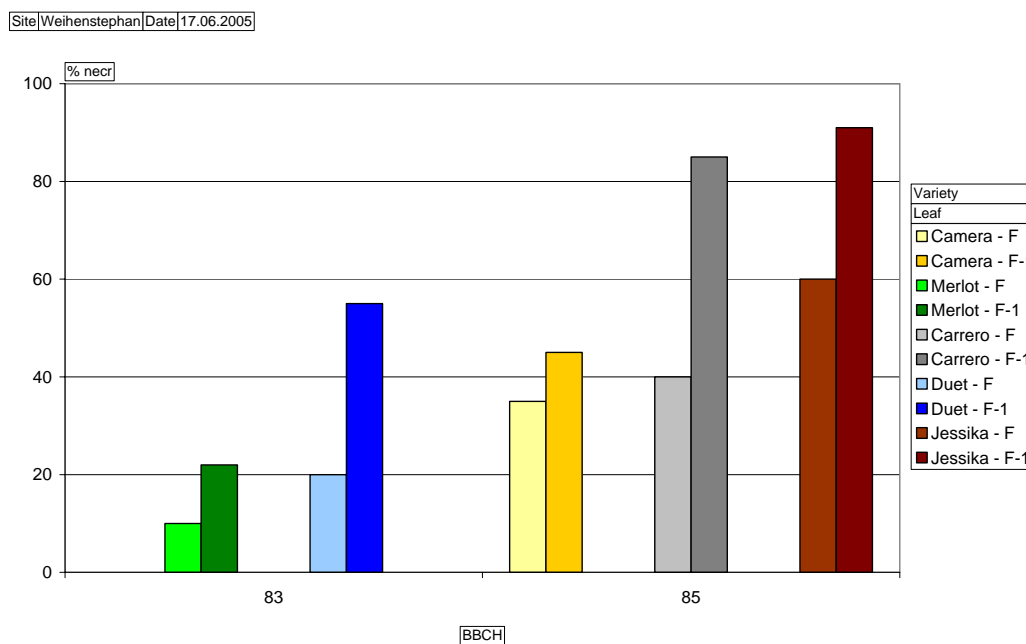
Figure 1. Comparison of % necrotic leaf area on the upper leaves at 17.06.2005 from different winter barley varieties. The varieties with the later growth stage (BBCH 85) show more severe symptoms.

Growth stage

For the further investigation of the influence of the growth stage, a trial in summer barley with different seed timings was carried out in Weihenstephan in 2005. The summer barley (var. Auriga) sown at 5.4.2005 (S1) and at 2.5.2005 (S2) shows similar development of symptoms (% necrosis) and disease (% Rcc) with 2 weeks delay for the late seed timing (figure 2). The onset of epidemics is for both seed timings at similar growth stages during grainfilling, about 2 weeks after heading. The relationship between maturity and symptom development was already observed in other trials (Formeyer *et al.*, 2001; Cromey *et al.*, 2004), but how epidemiology is influenced by this physiological factor and its significance for resistance and control needs further investigation.

Epidemics at one site 2001-2004

Since 2001 leaf spot trials have been conducted at the site in Zurnhausen. In all years the flag leaf was unrolled in the first week of May (Table 1). The first necrotic leaf spots on the upper leaves appeared between 12.05. and 04.06. so the onset of the epidemics varied over the time period of more than 3 weeks. Changes in weather, especially maximum global radiation, which are associated with the occurrence of physiological leaf spots (Obst *et al.*, 1995), appeared during this time. These kind of changes are typical for the region, but can not be directly related to causing the leaf spots. The breakdown of the crops was registered between 12.06. and 05.07. First sporulation of *Ramularia collo-cygni* on upper leaves was found from 12 days before until 2 days after breakdown. The time between the occurrence of the first

spots until breakdown compared to the time between flag leaf unrolled and sporulation of *Ramularia collo-cygni* indicates that the sooner *Ramularia collo-cygni* is found in the crop, the faster the breakdown of the crop follows the first spots.

Leaf F-1

| Sampling | Date | BBCH | |
		S1	S2
T1	16.06.2005	51	
T2	22.06.2005	59	39
T3	29.06.2005	73	53
T4	06.07.2005	75	57
T5	13.07.2005	83	65
T6	21.07.2005	91	83
T7	29.07.2005		87
T8	04.08.2005		89
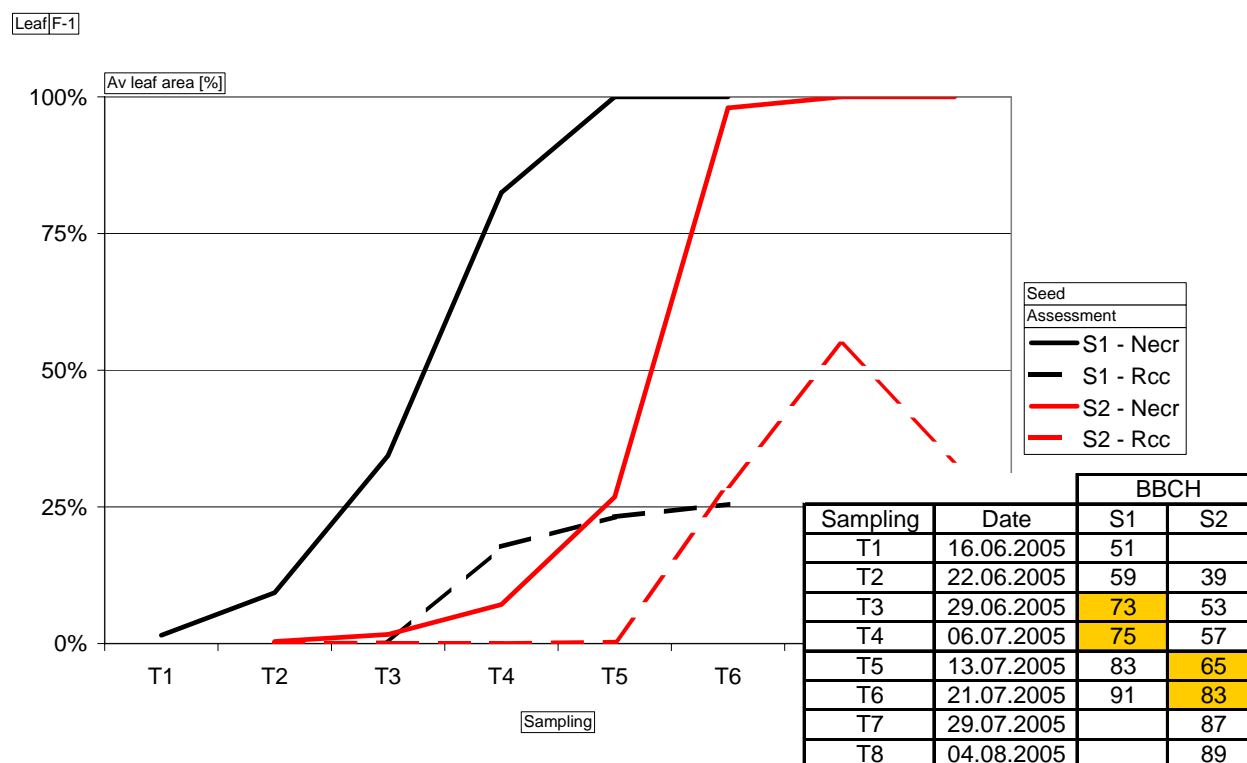
Figure 2. Summer barley (var. Auriga) sown at 5.4.2005 (S1) and at 2.5.2005 (S2) show similar development of symptoms (% necrosis) and disease (% Rcc) with 2 weeks delay for the late seedtiming. The onset is at the same growth stage (orange boxes).

Table 1. Comparison of *Rcc* epidemics on flag leaf and F-1 (flag leaf unrolled, first leafspots, breakdown, *Rcc* sporulation) in winter barley in Zurnhausen 2001-2004.

Year	2001	2002	2003	2004
BBCH 39	07.05.	03.05.	07.05.	05.05.
First spots	12.05.	27.05.	26.05.	04.06.
Breakdown	20.06.	20.06.	12.06.	05.07.
Sporulation of *Rcc*	21.06.	08.06.	10.06.	25.06.
Days from first spots to breakdown	39	24	17	31
Days from BBCH 39 to Rcc	45	36	34	51

Methods and Site
Observing the development of *Ramularia collo-cygni* by microscope is a simple and reliable method, but it lacks sensitivity since the pathogen can only be diagnosed after severe necrosis has already appeared (figure 3), sometimes only shortly before breakdown.

Different methods were employed to improve sensitivity and accomplish better knowledge of epidemics. Counting spores being washed off from leaves (figure 3) and relating the number to the development of necrotic leaf area and the area with sporulating *Ramularia collo-cygni* shows a strong relationship between the sporulation found on the leaf surface and the number of spores being washed off. Both follow the development of the necrotic leaf area with about 1 week delay. A greater number of spores as inoculum preceeding the first symptoms could not be found with this method.
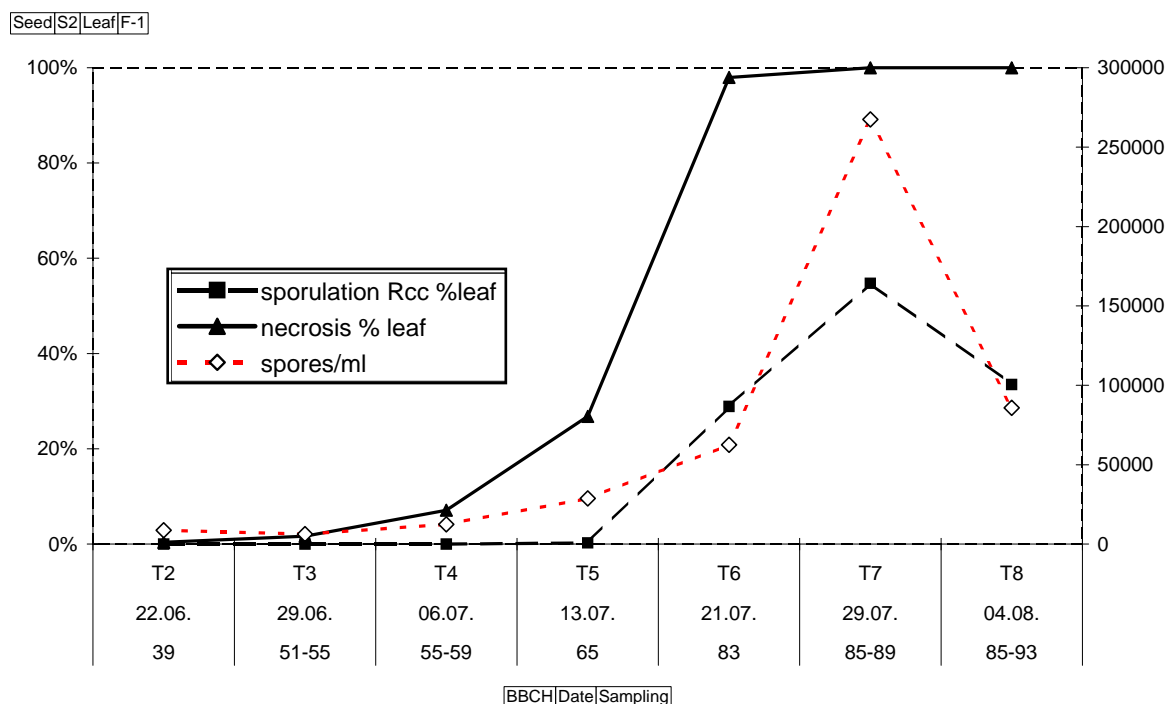
Figure 3. Number of spores being washed off from leaves (F-1) related to the development of necrotic leaf area (necrosis %leaf) and the area with sporulating *Ramularia collo-cygni* (sporulation Rcc %leaf)

Early detection of the fungus is possible with PCR diagnostics. In Weihenstephan in 2004 the flag leaf unrolled on 04.05., first necrotic spots on the F-1 were observed on the 09.06. and breakdown followed on 30.6. (table 2). Sporulation of *Ramularia collo-cygni* on F-1 was observed on the 20.6.. In 2005 the sporulation was detected earlier (06.06.) with earlier breakdown. Both years followed similar epidemiological patterns with the earlier onset of disease parameters in 2005. In both years PCR tests gave first positive results for *Ramularia collo-cygni* (leaf samples from 10.5.). But in 2005, F-1 were positive on 23.5. when first leaf spots appeared, 12 days earlier than in 2004, pointing to an earlier development of the disease.

Comparing sites in Weihenstephan and in Lohhof in 2005 showed that prediction of the epidemics by the results from the PCR tests remain difficult. Although F-1 was positive earlier in Lohhof than in Weihenstephan, disease progress was slower and breakdown later in 2005 (table 2).

Table 2. Comparison of *Rcc* epidemics on flag leaf and F-1 (flag unrolled - BBCH 39, first leafspots, breakdown, Rcc sporulation, PCR positive - site, F-1) in winter barley in Weihenstephan 2004 and 2005 compared to Lohhof 2005.

Site	Weihenstephan		Lohhof
Year	2004	2005	2005
BBCH 39	04.05.	15.05.	13.05.
First spots (F-1)	09.06.	23.05.	19.05.
Breakdown	30.06.	21.06.	23.06.
Sporulation *Rcc* (F-1)	20.06.	06.06.	12.06.
PCR + (Site)	10.05.	10.05.	08.05.
PCR + (F-1)	04.06.	23.05.	19.05.

The comparison of the epidemiological observations regarding sites, years and methods shows, that studying *Ramularia collo-cygni* by itself is not sufficient for explaining the development of leaf spots and to predict the sudden breakdown of crops.

The results from the variety trials and the influence of the growth stage unravel great impact of physiological and environmental factors for epidemiology. The production of the photodynamic rubellins in leaves, biologically active toxins (Miethbauer *et al.*, 2003), gives a possible link between infection with *Ramularia collo-cygni* and the observed reaction to environmental stress, especially global radiation. It is proposed that the toxin acts as pathogenicity factor (Heiser *et al.*, 2004). The significance of the toxin for the epidemics is subject of further investigations.

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A new direct PCR test detect *Ramularia collo-cygni* in barley

Peter Frei
Agroscope RAC, Swiss Federal Agricultural Research Station for Plant Production of Changins, P.O.Box 254 CH-1260 Nyon

Abstract: For a better understanding of the epidemiology of *Ramularia collo-cygni* B. Sutton & J.M. Waller, internal specific primers to the ITS1/2 rDNA regions were designed (Rcc1 and Rcc2) which amplify a 348 bp fragment. Direct–PCR, without any DNA purification steps, were performed during the whole period of vegetation of untreated winter and spring barley (respectively cv. Plaisant and cv. Celinka) to follow the epidemiology of the fungus. The plants were collected in two regions of Switzerland. Results show that, between growth stage DC 24 (4 tillers detectable) and DC 85 (grain content solid), *R. collo-cygni* is present in leaves, ears and awns after PCR amplification on homogenized plant juices. *R. collo-cygni* is therefore present during a long period of time in the plant which signifies that infection of barley takes place in autumn for winter varieties, where the fungus overpass the winter time and can infect spring barley very early in the season. The control of symptomless volunteers two months after harvest also showed the presence of the fungus in these plants and proves the "green bridge" between barley cultures.

As controls, other fungal pathogens and saprophytes isolated from barley, and also barley itself, were amplified with primers Rcc1 and Rcc2. No amplification products could be observed, this showing the high specificity of the primers.

Pathogenicity

Phytotoxins from *Ramularia collo-cygni*: Mode of action and contribution to pathogenicity

Ingrid Heiser[1] and Bernd Liebermann[2]

[1]*Institute of Phytopathology, Life Science Center Weihenstephan, Technical University of Munich, Am Hochanger 2, D-85350 Freising;* [2]*Intitute of Pharmacy, University of Jena, Neugasse 23, D-07743 Jena*

Abstract: Fungal and bacterial phytotoxins play an important role as virulence factors or even as determinants of pathogenicity in many plant-pathogen interactions. For *Ramularia collo-cygni* we were able to isolate several phytotoxic metabolites from the culture medium which could be identified as rubellins A-E. These red- or yellow-coloured anthraquinones showed toxicity on barley, the host plant of *R. collo-cygni*, as well as on tobacco, a non-host plant, and therefore can be addressed as non-specific phytotoxins. Symptoms induced by the rubellins appear as chlorotic and necrotic lesions which only develop in the light. The strong light-dependency of rubellin action can be explained by the photodynamic activity of these molecules. Analogous to cercosporin, a perylenequinone phytotoxin from *Cercospora* sp., the rubellins are activated by light and induce the formation of reactive oxygen species (ROS) after illumination. ROS like singlet oxygen, the superoxide radical anion, hydrogen peroxide and the hydroxyl radical are strong oxidants and induce the oxidative breakdown of membranes and pigments when the antioxidative capacity of the plant cell is exhausted. Therefore the rubellins may be responsible for the symptoms induced in barley leaves after infection with *R. collo-cygni*. The presence of rubellins in infected leaves further points to an important role for these toxins in the infection process.

Key words: phytotoxins, oxygen activation, fatty acid peroxidation, chlorophyll bleaching

Introduction

A great number of plant pathogenic fungi and bacteria are known to produce phytotoxins which contribute to disease symptom development in infected plants. Some phytotoxins are essential for pathogenicity, others only contribute to virulence of the toxin-producing pathogen but are not necessary for pathogenicity. In general, phytotoxins are divided into two groups: the host-specific toxins which affect only the host plants of the toxin-producing pathogen and the unspecific toxins that cause symptoms also on other plants.

The mode of action of phytotoxins includes impacts on enzymatic reactions, phytohormone activity or the destruction of membrane semipermeability by forming pores in the plant plasma membrane. Several phytotoxins exert their toxicity by the activation of oxygen leading to the production of reactive oxygen species (ROS) in the plant cell (Heiser *et al.*, 1998). ROS like the superoxide radical, the hydroxyl radical, hydrogen peroxide and singlet oxygen are toxic to cells and react rapidly with most organic molecules like fatty acids, proteins and nucleic acids. When ROS are produced within the cells the detoxification system may be overloaded and oxidative processes will take place. The peroxidative breakdown of unsaturated fatty acids is induced by hydroperoxy radicals derived from superoxide as well as by OH-radicals and also by singlet oxygen which directly produces lipid

hydroperoxides (Elstner, 1982). For a review of the formation and activity of ROS see Heiser & Elstner (1998).

Phytotoxins from **Ramularia collo-cygni**

First experiments revealed that ethylacetate extracts from culture filtrate from *R. collo-cygni* showed phytotoxic activity on barley leaves (figure 1). In the following we were able to isolate the phytotoxic compounds from the culture medium, to identify their chemical structure and to elucidate their biochemical mode of action. These results will be briefly summarised in the following chapters.
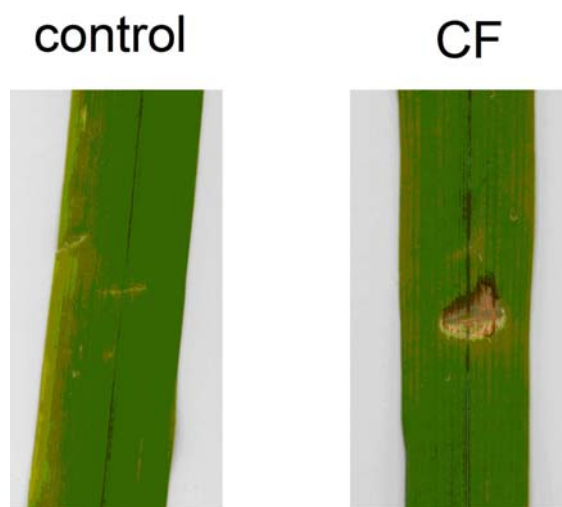
Figure 1. Symptoms induced on barley leaf segments by application of an extract (EtAc) from culture filtrate from *R. collo-cygni*. CF = culture filtrate.

Chemical structure

Isolation of the phytotoxic compounds and investigations on their chemical structure revealed that the phytotoxins produced by *R. collo-cygni* belong to the group of rubellins, which were hitherto described only for *Mycosphaerella rubella*, the causal agent of a necrotic spot disease in the medicinal plant *Angelica sylvestris* (Arnone *et al.*, 1986, 1989). The rubellins are a group of yellow or red coloured anthraquinones and we were able to identify the rubellins A, B, C, D and the novel compounds dehydro D and E as phytotoxic compounds from *R. collo-cygni* (Miethbauer *et al.*, 2003, 2006, Heiser *et al.*, 2003, 2004). Recent studies revealed that the rubellins are synthesised via the polyketide pathway (Miethbauer *et al.*, 2006). Figure 2 shows the chemical structure of rubellin D.
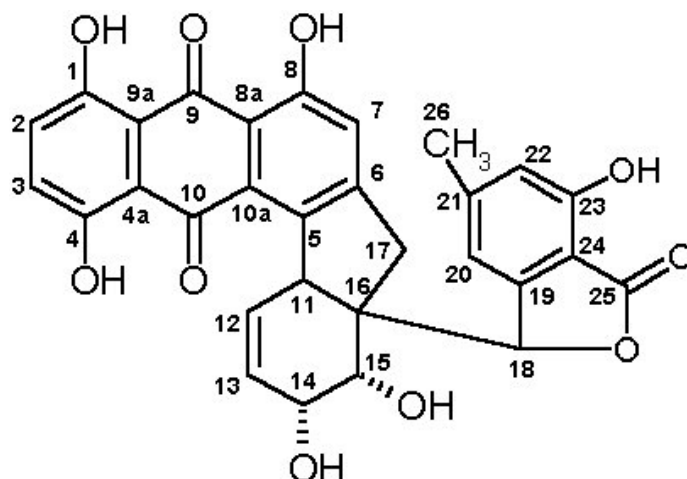
Figure 2. Chemical structure of rubellin D.

Symptoms

When barley leaves were treated with rubellin B or D, 2 days after treatment severe chlorotic and necrotic lesions occurred (Heiser *et al.*, 2003, figure 3). These symptoms only appeared when toxin-treated leaves were incubated under illumination, no necrosis could be observed in leaves incubated in the dark. Symptoms were also induced on tobacco leaves proving that rubellins are non-hostspecific phytotoxins (Heiser *et al.*, 2004). Also in the tobacco experiments phytotoxicity of the rubellins strongly depended on light. For several phytotoxins it was shown that the induction of symptoms is light-dependent and due to toxin-mediated oxygen activation (Heiser *et al.*, 1998, Daub and Ehrenshaft, 2000). This mechanism proved to be true also for the rubellins and will be described in more detail in the next paragraph.

Figure 3. Symptoms induced by rubellin B (0.3 mM) on a barley leaf segment after incubation for 48 hours in the light.

Photodynamic activity

For some toxins the light dependency of phytotoxic action can be explained by their photodynamic activity. In this kind of reaction a toxin is physically activated by light and transfers its excitation energy in a photodynamic type II reaction to oxygen, triggering the

formation of singlet oxygen. Singlet oxygen ($^1O_2$), in contrast to atmospheric oxygen, is not subject to the spin rule and reacts rapidly with most organic molecules, especially with double bonds, producing hydroperoxides (Foote, 1976). Photodynamic reactions not involving physical transfer of excitation energy but undergoing charge separation within the excited pigment are called photodynamic reactions type I (Elstner, 1982). In a type I reaction an electron is transferred from the toxin to oxygen and superoxide is formed. The mechanisms of photodynamic oxygen activation are summarised in figure 4.

$$P \xrightarrow{\;h\nu\;} P^* \longrightarrow P + {}^1O_2 \;\text{(II)}$$

$$P^* \downarrow \quad + O_2$$

$$^+P_- \longrightarrow {}^+P + O_2{}^{\cdot-} \;\text{(I)}$$
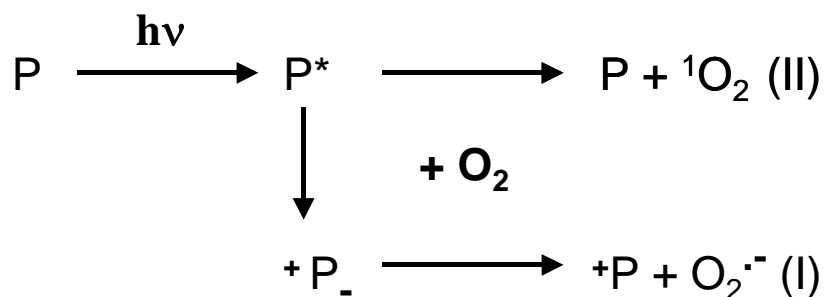
Figure 4. The mechanisms of photodynamic oxygen activation. P = pigment; I, II = type I or type II reaction.

An example of a fungal toxin which induces ROS formation after illumination is cercosporin, a perylenequinone produced by several phytopathogenic *Cercospora* species e.g. *C. beticola* and *C. kikuchii* (Assante *et al.*, 1977, Daub 1982). Several groups proved the formation of singlet oxygen (Daub and Hangarter, 1983, Youngman *et al.*, 1983) as well as of superoxide (Daub and Hangarter, 1983) by cercosporin by type II and type I photodynamic reactions, respectively.

Our studies showed that the rubellins are also able to perform a photodynamic reaction inducing the light-dependent formation of reactive oxygen species (Miethbauer *et al.*, 2003, 2006, Heiser *et al.*, 2003, 2004). Via this mechanism the rubellins induced the peroxidation of α-linolenic acid in a biochemical model system as well as in toxin-treated leaves from barley and tobacco (Heiser *et al.*, 2004). The application of ROS-scavengers showed that singlet oxygen quenchers were able to inhibit rubellin-induced fatty acid peroxidation. Therefore we assume that mainly singlet oxygen is produced by rubellins in a photodynamic type II reaction.

Contribution to pathogenicity
Several factors point to an involvement of the rubellins in the infection process. First, we were able to detect both rubellin B and rubellin D in barley leaves infected with *R. collo-cygni* (Miethbauer *et al.*, 2003). Concentrations in infected leaves reached levels high enough to be active in the bioassay.

Second, via the biochemical mode of action of the rubellins symptoms after *R. collo-cygni*-infection might be explained. Rubellins were shown to induce fatty acid peroxidation (see above) which finally leads to membrane destruction and necrosis. Reactive intermediates formed during this peroxidation process can oxidatively attack pigments leading to bleaching and chlorosis. Therefore, both necrotic and chlorotic symptoms after infection can be explained by rubellin action.

Summary

Figure 5 gives a model for the involvement of rubellins in symptom expression after infection of barley with *R. collo-cygni*.
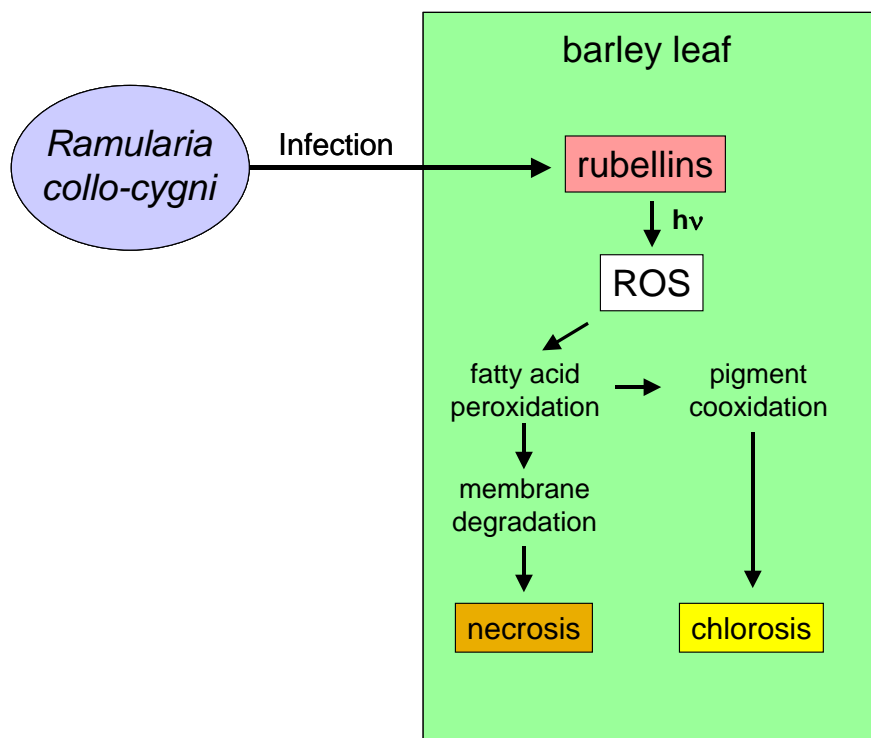
Figure 5. Model for the involvement of rubellins in the infection process. ROS = reactive oxygen species.

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Biosynthesis of the rubellins, produced by the phytopathogenic fungus *Ramularia collo-cygni*

Bernd Liebermann [1]**, Wolfgang Günther** [2]**, Sebastian Miethbauer** [1]

[1] *Institute of Pharmacy, University of Jena, Neugasse 23, 07743 Jena, Germany, email: bernd.liebermann@uni-jena.de*
[2] *Institute of Organic and Macromolecular Chemistry, University of Jena, Humboldtstraße 10, 07743 Jena, Germany*

Abstract: The phytopathogenic fungus *Ramularia collo-cygni* (Sutton and Waller) produces anthraquinone derivatives (rubellins) with photodynamic activities. Incorporation experiments with [1-^{13}C]-acetate and [2-^{13}C]-acetate, respectively, reveal that these rubellins were biosynthesised via the polyketide pathway. The labelling pattern in the anthraquinone derivatives after feeding with [U-^{13}C$_6$]-glucose proved the fungal folding mode of the poly-β-keto chain.

Key words: Rubellins; Anthraquinones; Biosynthesis; Folding mode; Polyketide pathway; Phytotoxin

Introduction

Ramularia collo-cygni (Sutton & Waller) is the causal agent of leaf spot disease of barley and other cereals (Huss *et al.*, 2005). Recently it was revealed that different anthraquinone derivatives are involved in this phytopathogenic event (Heiser *et al*., 2003, 2004; Miethbauer *et al*., 2003). In order to clarify on which pathway these rubellins would be biosynthesised we applied feedings with ^{13}C-precursors (Miethbauer *et al*., 2006).

Material and methods

Fermentation procedure and isolation conditions were carried out as described by Miethbauer et al. (2006). In the feeding experiments with ^{13}C-precursors we used a culture medium stabilised by a Soerensen phosphate buffer (66 mM, pH 5.4) in order to get a sufficient amount to rubellin B. 2 g sodium [1-^{13}C]-acetate and 2 g sodium [2-^{13}C]-acetate (both 99% ^{13}C), respectively, were added to 10 x 100 ml of culture medium. Feeding with glucose (without sucrose) was performed with 4% (w/w) [U-^{13}C$_6$]-D-glucose (99% ^{13}C) diluted with unlabeled glucose (altogether 25 g in 25 x 100 ml of culture medium). In each case rubellin B and D were isolated after 14 days.

Results and discussion

Incorporation of ^{13}C labelled acetate into rubellin B and D, respectively, exactly reveals that biosynthesis proceeds on the polyketide pathway (Figure 1).
Incorporation of uniform labelled ^{13}C acetate (from uniform labelled glucose) into the rubellins results in a specific ^{13}C-^{13}C coupling pattern showing the exact incorporation position of these intact acetates. The initial ring of aromatic polyketides biosynthesised by

streptomycetes consists of three intact acetate units (mode S folding), in contrast, in fungi only two units (mode F folding) are found (Thomas, 2001). Red coloured bonds (Figure 2) in the initial ring demonstrate a coupling pattern which proves that *R. collo-cygni* folded rubellins via mode F, too.
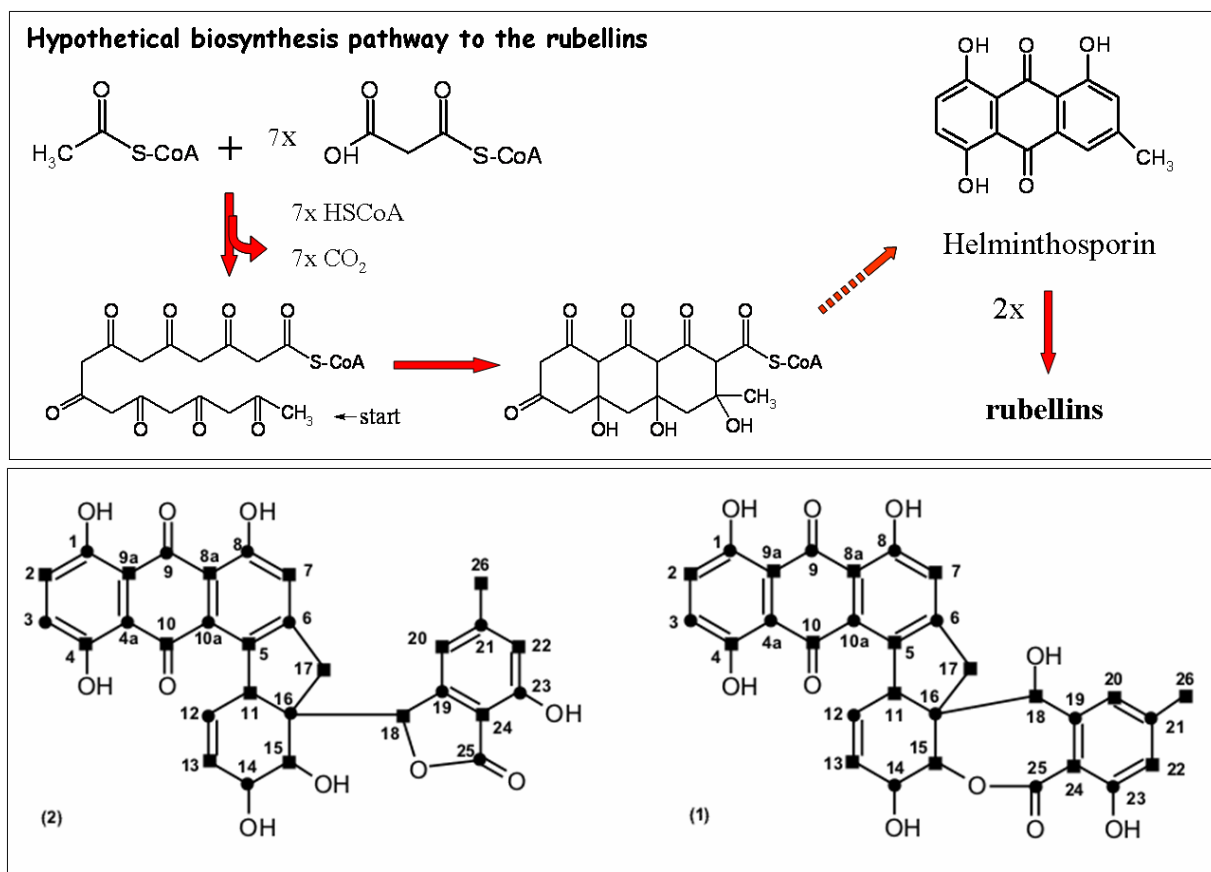
Figure 1. ^{13}C-labelling patterns after incorporation of sodium [1-^{13}C]-acetate and sodium [2-^{13}C]-acetate by rubellin B (1) and rubellin D (2). Increased ^{13}C-level caused by sodium [1-^{13}C]-acetate (●) and sodium [2-^{13}C]-acetate (■).
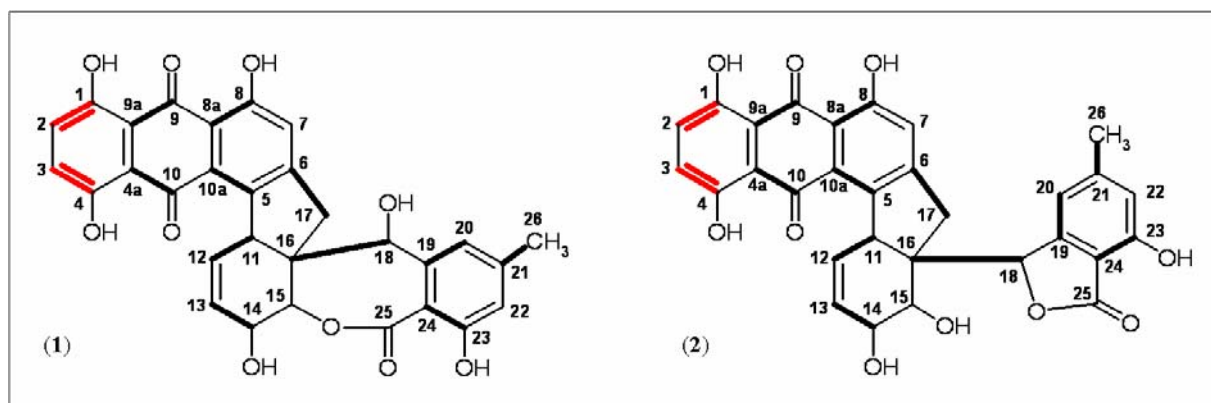
Figure 2. Incorporation of [U-[13]C$_6$]-glucose into rubellin B (1) and D (2), 4% (w/w) diluted with unlabelled glucose; bold bars: carbon atoms involved in [13]C-^{13}C coupling.

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The phytopathogenic fungus *Ramularia collo-cygni* produces different anthraquinone derivatives (rubellins) with photodynamic activity

Sebastian Miethbauer [1]**, Wolfgang Günther** [2]**, Ingrid Heiser** [3] **and Bernd Liebermann** [1]
[1] *Institute of Pharmacy, University of Jena, Neugasse 23, 07743 Jena, Germany, email: bernd.liebermann@uni-jena.de*
[2] *Institute of Organic and Macromolecular Chemistry, University of Jena, Humboldtstraße 10, 07743 Jena, Germany*
[3] *Institute of Phytopathology, Technical University of Munich, Am Hochanger 2, 85350 Freising-Weihenstephan, Germany*

Abstract: The anamorph fungus *Ramularia collo-cygni* (Sutton & Waller) causes a leaf spot disease on barley and other Poaceae. Rubellins as non-host-specific toxins contribute to symptom development in the infected plant. In addition to the rubellins B, C and D, we isolated the yellow coloured anthraquinone derivative rubellin A from mycelium and culture filtrate. Furthermore, two novel compounds called rubellin E and 14-dehydrorubellin D were isolated and elucidated in their structure. In comparison to the others, rubellin A shows increased photodynamic oxygen activation. The ability to produce rubellins is not limited in the anamorph genus *Ramularia* to *R. collo-cygni*.

Key words: Rubellins; Anthraquinones; Photodynamic activity; Phytotoxin

Introduction

At the end of the 1980s a novel leaf spot disease appeared on barley in Central Europe (Huss *et al*., 1987). It was revealed that the phytopathogenic fungus *Ramularia collo-cygni* (Sutton & Waller) is the causal agent of this disease called "Ramularia Leaf Spot Disease". Due to early ripening of infected plants, the pathogen causes yield losses of up to 20%. It is known that phytopathogenic fungi produce secondary metabolites being involved in a disease emergence. Therefore, we searched for such phytotoxins in *R. collo-cygni*.

Material and methods

Cultivation, isolation and investigation of photodynamic activities are described in Heiser *et al*. (2003, 2004) and Miethbauer *et al*. (2003, 2006).

Results and discussion

The rubellin spectrum of *Mycosphaerella rubella* described by Arnone *et al*. (1989) was completely proved in the phytopathogenic fungus *Ramularia collo-cygni*, in addition three novel related anthraquinone derivatives (rubellin E, F, and 14-dehydrorubellin D, Table 1) were elucidated in their structure (Miethbauer *et al*., 2006, Figure 1). Radical oxygen species

(ROS) generated by all rubellins may contribute to the symptoms of the leaf spot disease of barley and other cereals (Heiser *et al.*, 2004, Figure 2 and 3).

Table 1. Physicochemical properties of rubellins.

compound	rubellin A	rubellin B	rubellin C	rubellin D	14-dehydro rub. D	rubellin E	rubellin F
HRESIMS [*m/z*]	525.1193	541.1158	525.1188	541.1154	539.1002	559.1213	543.1271
empirical formula	$C_{30}H_{21}O_9$	$C_{30}H_{21}O_{10}$	$C_{30}H_{21}O_9$	$C_{30}H_{21}O_{10}$	$C_{30}H_{19}O_{10}$	$C_{30}H_{23}O_{11}$	$C_{30}H_{23}O_{10}$
specific optical rotation (MeOH) [°] / [g/100ml]	310°/c0.1	350°/c0.1	220°/c0.1	350°/c0.1	1600°/c0.01	2800°/c0.01	-
UV/Vis λ $_{max}^{MeOH}$ nm (log e)	443 (4.08)	499 (4.25)	443 (4.08)	499 (4.20)	500 (4.11)	501 (3.97)	449
analyt. HPLC [min]	18.4	19.5	15.6	16.7	17.9	5.5	4.5
TLC RP-18 (R$_f$)	0.17	0.10	0.24	0.19	0.16	0.48	0.52
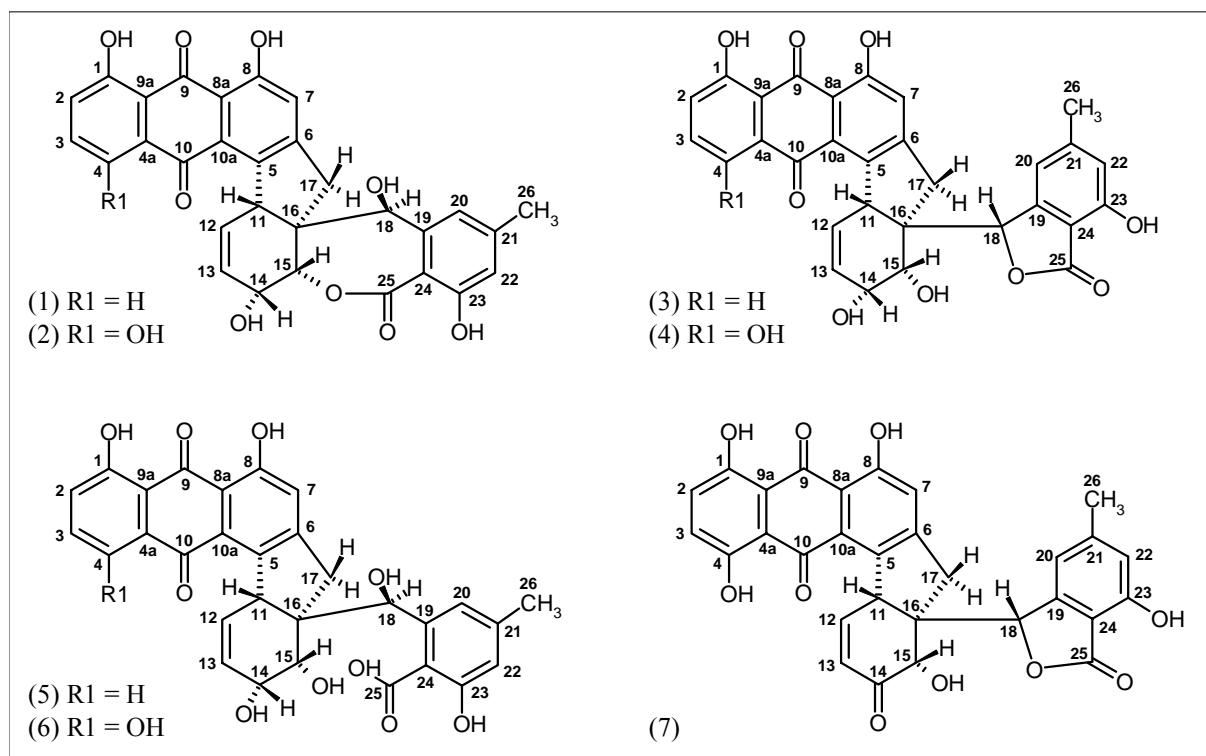
Figure 1. Structures of rubellin A (1), B (2), C (3), D (4), E (5), F (6), 14-dehydrorubellin D (7).
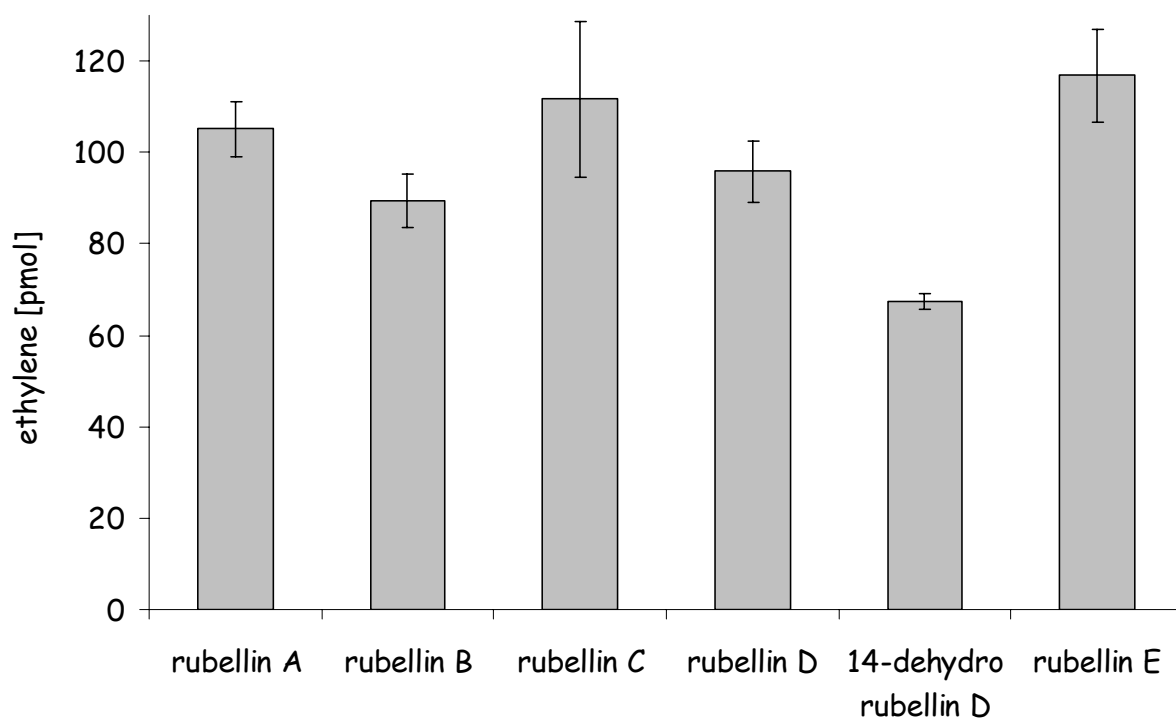
Figure 2. Ethane formation from α-linolenic acid as indicator of singlet oxygen in a photodynamic reaction.
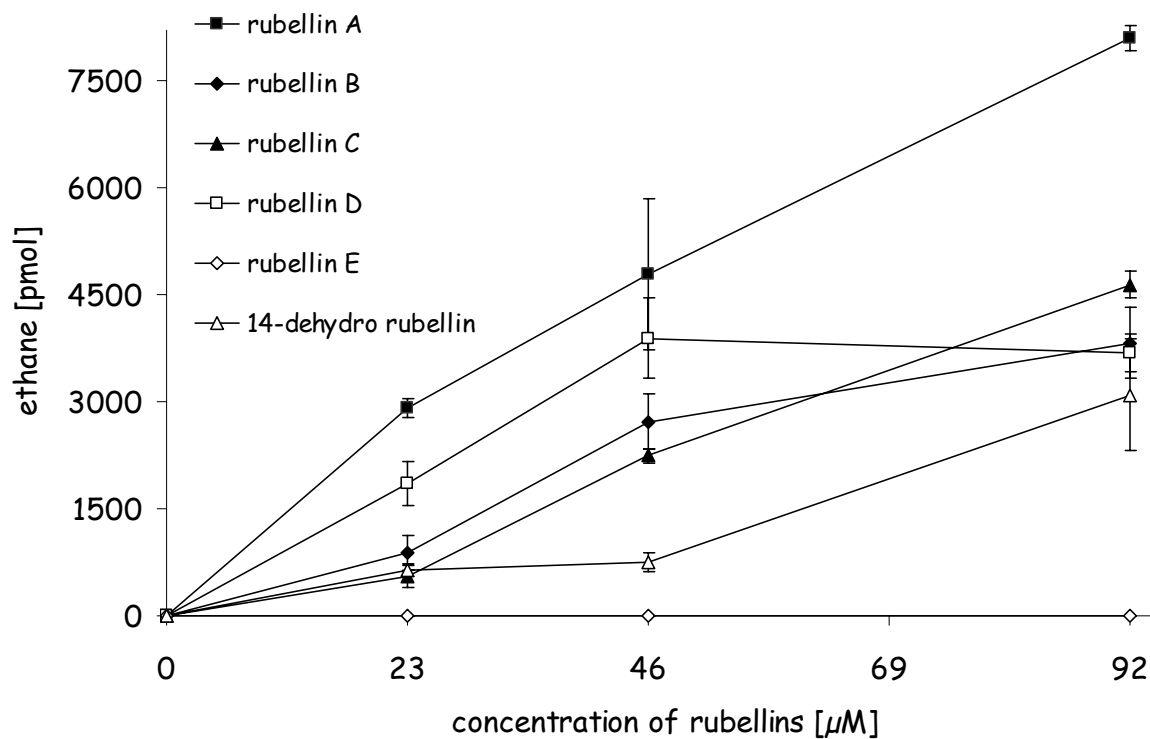
Figure 3. Ethylene formation from α-keto-4-thiomethyl butyric acid as indicator of oxygen radicals in a type I photodynamic reaction (Heiser *et al.*, 2004).

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Resistance

Field screening in Norway for resistance to *Ramularia collo-cygni* in old and new barley material

Lars Reitan[1] and Saideh Salamati[2]
[1] Graminor AS, branch Holthe, N-7650 Verdal, Norway. [2] Midt-Norsk Plantevern AS and Bioforsk Central-Norway, N-7500 Stjørdal, Norway.

Abstract: The symptoms of Ramularia Leaf Spot of Barley (RLSB or Rcc severity.) caused by the fungus *Ramularia collo-cygni* (Rcc) has been known for at least two decades in the central Norway cereal producing region. The agent itself was on the other hand recognized as late as1999. The regional barley breeding programme had to face the challenges of this fungus, and a rather comprehensive testing programme was started. A research project was initiated as well with some regional money support in order to solve some of the problems with isolation, inoculation and testing for resistance. This report addresses attention to the field testing part of the project. Both fungicide experiments and nurseries trials were conducted. Great significance of fungicide treatments on both yield and quality implies that the Rcc fungus can cause considerable damage. Variety variation in tolerance is shown. Despite testing several thousands barley genotypes, so far no complete resistance is observed. Other diseases have influence on the development of Rcc on the plant, and make confusion in assessing tolerance levels. There is some indication that early mildew attacks cause partial induced plant resistance to Rcc.

Key words: *Hordeum vulgare* L., *Ramularia collo-cygni* Sutton & Waller, Field testing.

Introduction and background

In the Central Norway (Trøndelag) region barley is important, and counts for about 20% of the total Norwegian barley production. However, the climate is quite different from the main cereal producing area (south-east Norway) through lower temperature, higher precipitation and longer leaf wetness durations (marine climate).

Typical leaf spotting (RLSB) symptoms caused by *Ramularia collo-cygni* (*R. collo-cygni* or Rcc) was observed in this region in the 1980s, although incorrectly connected to other fungi. Field experiments treated with and without fungicides (propiconazoles) reduced the symptoms. It was clear that the most effective time of treatment was late in plant development (after heading (Reitan, 1996)). At that point of time the *Ascochyta* fungus was blamed for the damages. In 1999, the connection between the symptoms and the *Ramularia collo-cygni* fungus was stated (Huss & Sachs, 1998, Salamati & Reitan, 2000). From then barley breeding material was scored for Rcc-severity (RLSB), and in some years a yield loss of at least 1 ton/ha was observed for the susceptible check variety.

Material and methods

Field experiments with fungicides and varieties
In early 1990s, field experiments were conducted and the effect of fungicides (treated contra untreated) on RLSB was studied on different varieties. Results of these experiments showed a substantial yield loss explained by Ramularia leaf spot attack (Reitan, 1996). These results were also confirmed by Salamati (2003) in a different trial series and spraying regimes which gave a mean of about 11% yield loss. In 2005, a new series of field trials was conducted in order to confirm the results and to look at differences in variety reaction to yield loss and quality implications. The experiment was a split plot design with 9 commercial barley varieties (of which 6 were six-rowed and 3 two-rowed), a fungicide regime (untreated, 'Stereo' at BBCH 30 and 'Stereo' (BBCH 30) +'Stratego' at BBCH 45) and a fertilizer regime (with and without split fertilizing). All together the experimental design counted for 54 different entries. Six locations scattered in cereal growing region of central Norway were involved in this series. 'Stereo' is a combination of propiconazole (62.5 g/l) and cyprodinil (250 g/l) and 'Stratego' a combination of propiconazole (125 g/l) and trifloxystrobin (125 g/l).

Observations and RLSB nurseries
Since 2000 we have established field nurseries for RLSB observations on breeding material, potential resistance sources and material from other breeders and collections.
The barley lines were sown in 'hill plots' at distances of 40 x 40 cm in grid blocs of 12 x 12 lines and with borders. The sowing equipment was a modified Wintersteiger Seedmatic. In majority of the years, three replicates were used. Due to the humid-keeping soil (silt- sand) and the humid climate the natural infection occurred at a high level each year. In order to avoid other leaf diseases we chose fields that were not grown by barley the last to years.

Nordic GeneBank (NGB) material
In 2004 and 2005 a Nordic Gene Bank (NGB) collection of Nordic old and younger varieties were tested in two environments in the region Trøndelag. A set of 213 NGB lines and breeding material from other sources were tested together in our hill plot testing system.

Results and discussions

Fungicide treatment experiments
One problem in studying yield and quality loss due to fungi attacks are the risk of more than one disease at the same time, and problems with the interpretation of the results. Therefore two different fungicides were used, and one of them has minor effect on *R. collo-cygni* but it is effective against other diseases like mildew, scald and net blotch, while the combination of these two is very effective against *R. collo-cygni*. The results showed severe *R. collo-cygni* attacks without fungicides and a very good control when using them. As much as 22% yield increase for eliminating all diseases in a certain variety was shown (average of six experimental fields), and even more in single fields. The less effective fungicide ('Stereo') treatment gave a yield gain of 8%, and thus the *R. collo-cygni* effect is at least 14%. The same great effect was also shown for *R. collo-cygni* attacks and quality measures like hectolitre weight (Hlv), (Figures 1, 2 and 3).
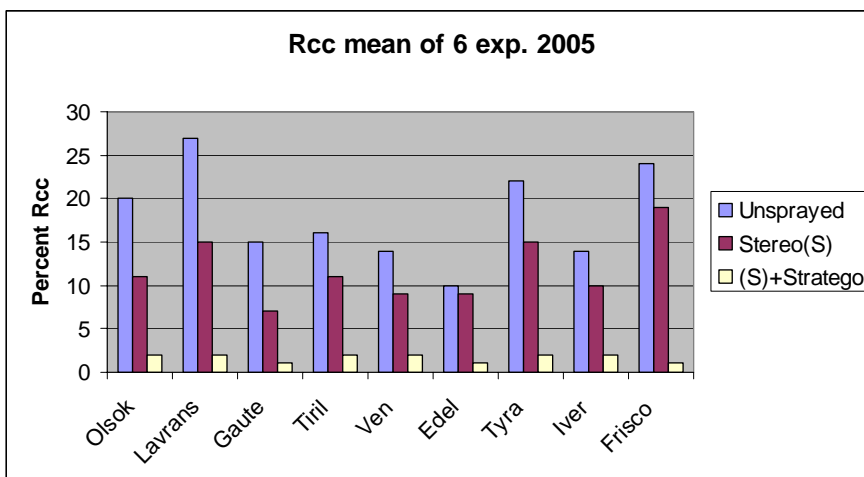
Figure 1. Ramularia Leaf Spot of Barley severity on six spring barley varieties untreated or treated with fungicides. The three varieties far right are 2-rowed.
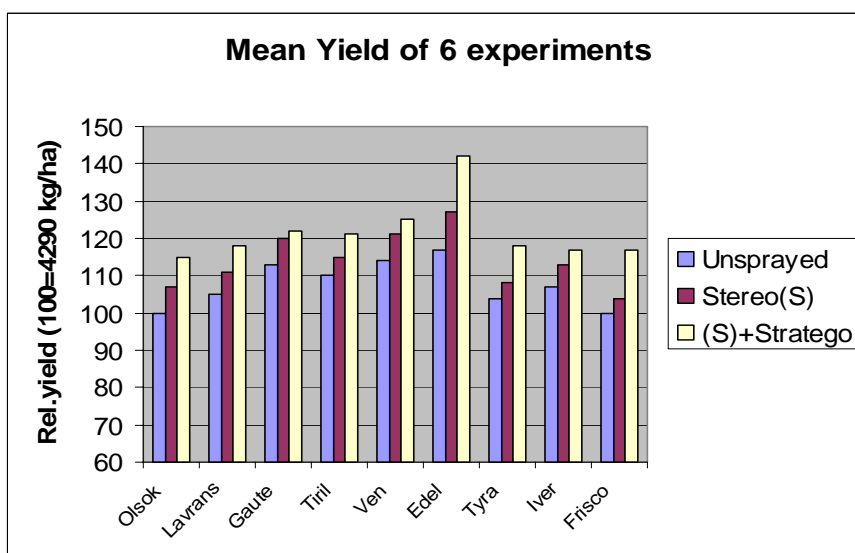
Figure 2. Yield results (relative values) of varieties untreated or treated with fungicides.
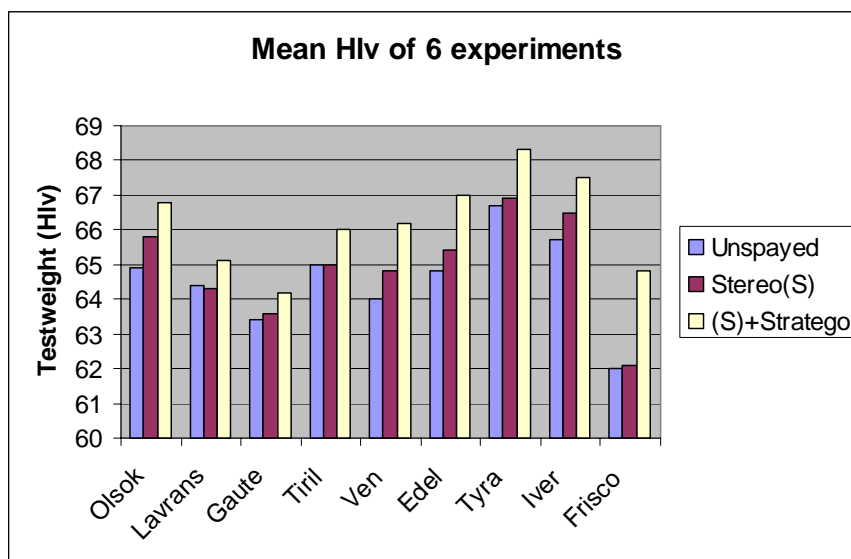
Figure 3. Quality assessment results (hectolitre weight) of varieties untreated or treated with fungicide.

Nurseries with hill plot testing

Here only small examples of the results can be presented.

Figure 4 shows the variation in RLSB score for some of the material tested in the period. The mean, maximum, minimum and mean deviation for the scores are shown for many hillplot field experiments and the mean deviations are small compared to the RLSB variation.

Several thousands genotypes have been tested since the start, and so far, no barley line or variety completely resistant to *R. collo-cygni* is found.
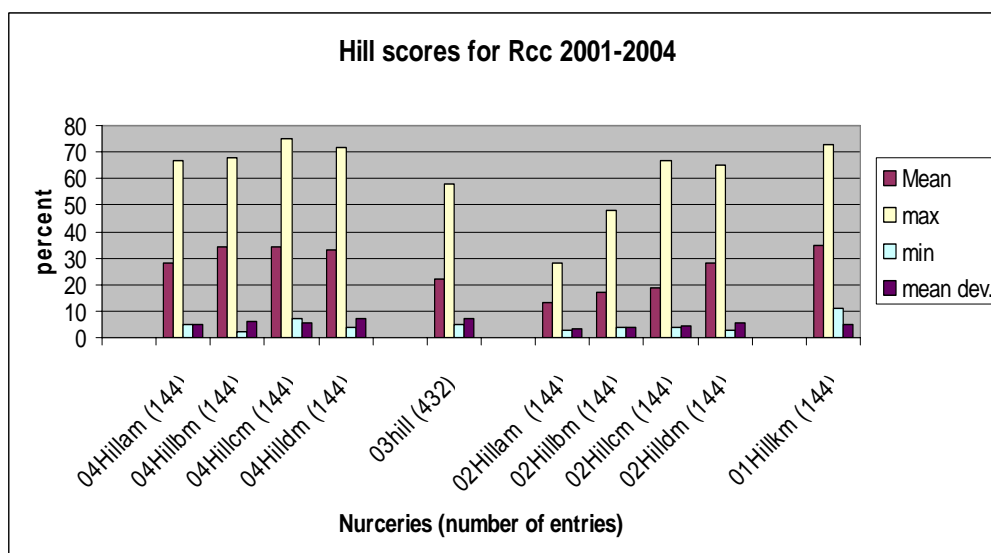
Figure 4. Mean, maximum, minimum values as well as mean deviation of Rcc (RLSB) scores in different fields in the years 2001 to 2004. Number of entries in each field is given in parentheses.

In Figure 5, varieties/lines (18) tested for the year 2002 to 2005 are shown.
The statistical variety effect over these years is highly significant (*P* %<0,01).
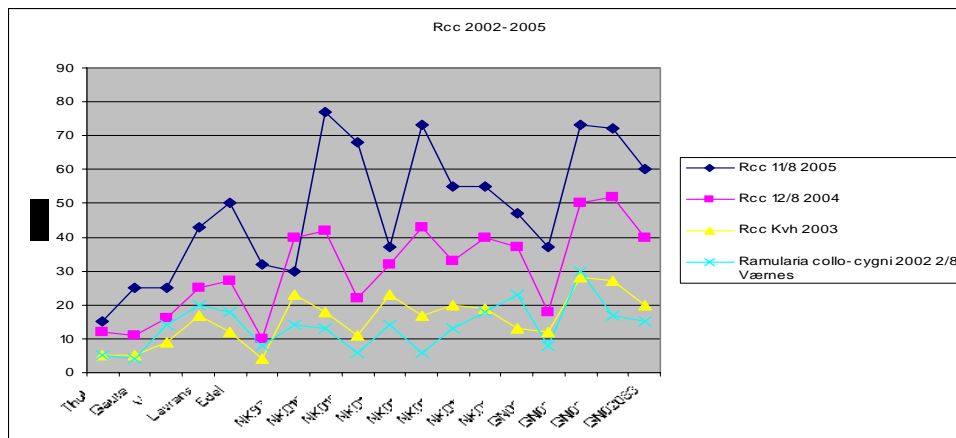
Figure 5. *R. collo-cygni* severity scores for 18 varieties/breeding lines over the years 2002 to 2005.

Comparing hillplot testing and normal field experiments

In 2005 a series of four ordinary field testing experiments (25 varieties) were scored for *RLSB*. The same material was tested in hill plot nurseries as well and scored for *RLSB*. The correlation between the mean field results and hill plot results was found to be r = 0,744. It is obvious that a lot of environmental effects are active both in the infection and the assessment processes.

NGB material

Similar nurseries in other Scandinavian countries failed to give *RLSB* symptoms. Results from two fields in each of the two years (central Norway) were computed for the varieties adjusted values around zero as means. They are shown sorted in increasing *R. collo-cygni* severity order in figure 6. Standard deviation for each entry is included as well. Corrections due to environmental factors as earliness and mildew attacks are computed. The most negative values (close to -20) are the best varieties.
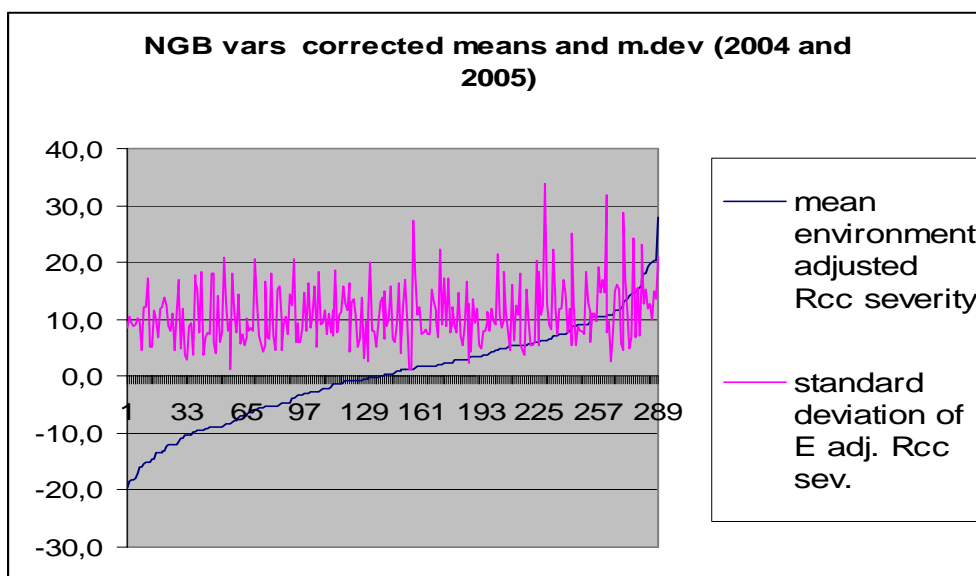
Figure 6. Adjusted values (to zero as mean) and standard deviation for NGB entries (and others) to Ramularia Leaf Spot (Rcc) severity (sorted). Data is form two experiments over the years 2004 and 2005.

Ramularia versus other diseases

Although the environment has considerable effect on *R. collo-cygni* occurrence and development, the genetic variability in barley has the greatest significance. There is a large variation between cultivars in tolerance to *R. collo-cygni*, but difficulties with the scoring due to other diseases are present. Especially mildew (*Blumeria graminis, Bg*) and net blotch *(Pyrenophora teres*, *Pt*) can cause problems in late sowing. Antagonism between Ramularia leaf spots and other diseases, like dwarf leaf rust (*Puccinia graminis)* and scald (*Rhynchosporium secalis*) are reported (Sachs: http://www.bba.de/inst/a/ramularia/ , Salamati, 2002)

In 2005, the NGB-material earlier described was assessed for mildew attacks (*Bg*) as well, in one field (Værnes, Væ). In the field Holthe there were very limited Bg attacks. Grouping the lines according to the mildew-scoring gave the 17 groups shown in Figure 7. The numbers below the figure represent the number of lines in each group. The *R. collo-cygni* attack in the Væ-field decreased with increasing Bg-attacks, and much more than expected due to leaf space occupied by Bg. The *R. collo-cygni* attacks in the Holthe-field showed no such decrease, except for the first Bg groups from 0 to 1 (percentage of Bg). These findings indicate that early mildew attacks can be antagonistic and may even cause a partial induced resistance to *R. collo-cygni*. Even small attacks (1%) caused a substantial reduction in *R. collo-cygni* severity score.

These findings lead to a practice of correcting the *R. collo-cygni* severity scores for present Bg scores, and was made by covariance correction in the statistical analysis procedure. Figure 8 and 9 show tested breeding material with and without corrections for different Blumeria groups.
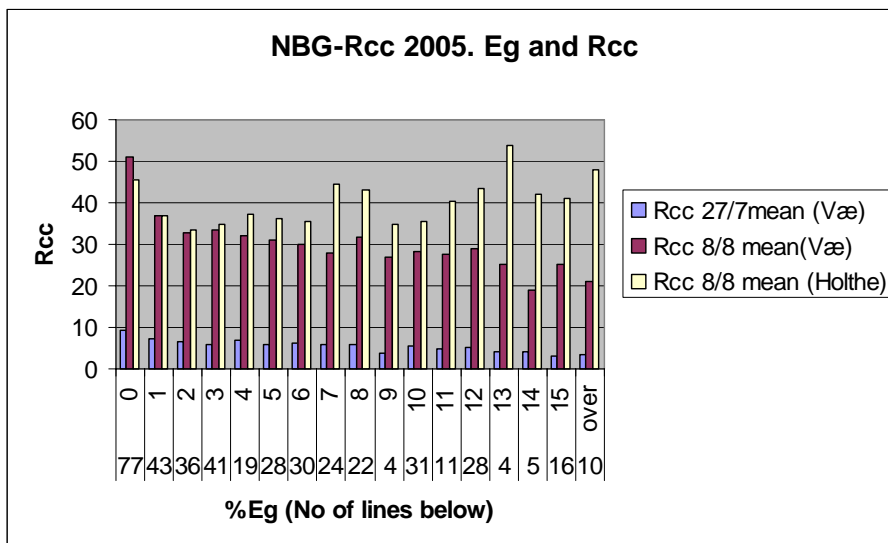
Figure 7. Groups of lines with increasing *Blumeria graminis* attacks and the corresponding *Ramularia collo-cygni* severity score in two different field nurseries. Lower line presents the number of lines in each group.
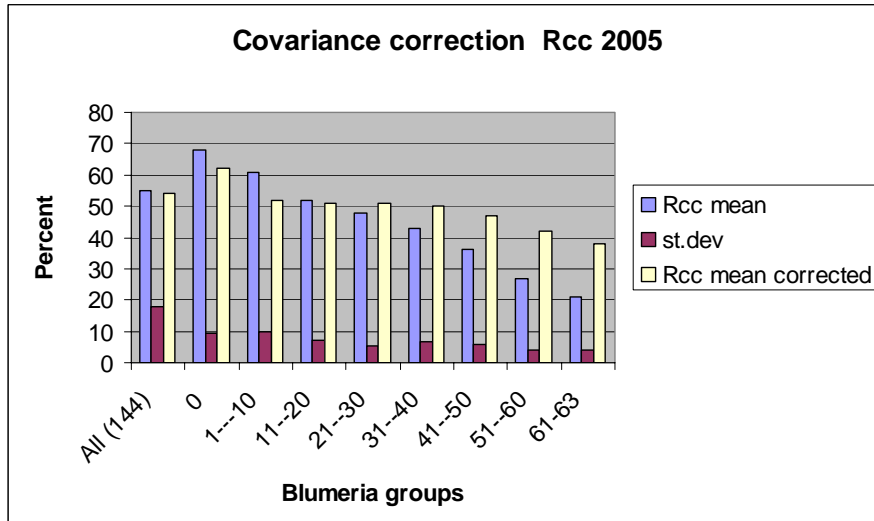
Figure 8. Groups of lines with increasing *Blumeria graminis* attacks and the corresponding *Ramularia collo-cygni* severity scores uncorrected and covariance corrected for *Blumeria graminis* .
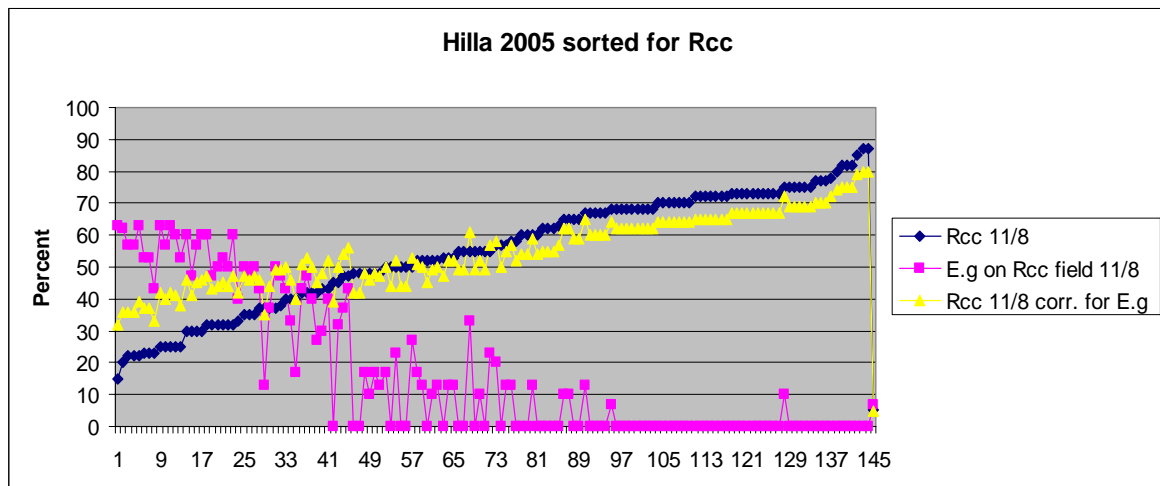
Figure 9. Increasing *Ramularia collo-cygni* severity scores uncorrected and covariance corrected for *Blumeria graminis* and the corresponding *Blumeria graminis* attacks in Graminor breeding material.

Breeding barley material resistant to *Blumeria graminis* (Bg) should give a more 'correct' view of the Ramularia 'resistance' or tolerance. According to the first two groups shown in Figure 7, the Bg resistance gene(s) itself would account for a higher susceptibility to *R. collo-cygni*. Looking at Bg resistant material (given zero attacks) over years, the tolerance pattern are similar for all years (Figure 10 and 11). Most of this material has ml-o resistance, but a limited number have other Bg resistance sources, but they do not differ from the fact that *R. collo-cygni* get high scores. Still there is significant variation in *R. collo-cygni* tolerance between the Bg-resistant materials.
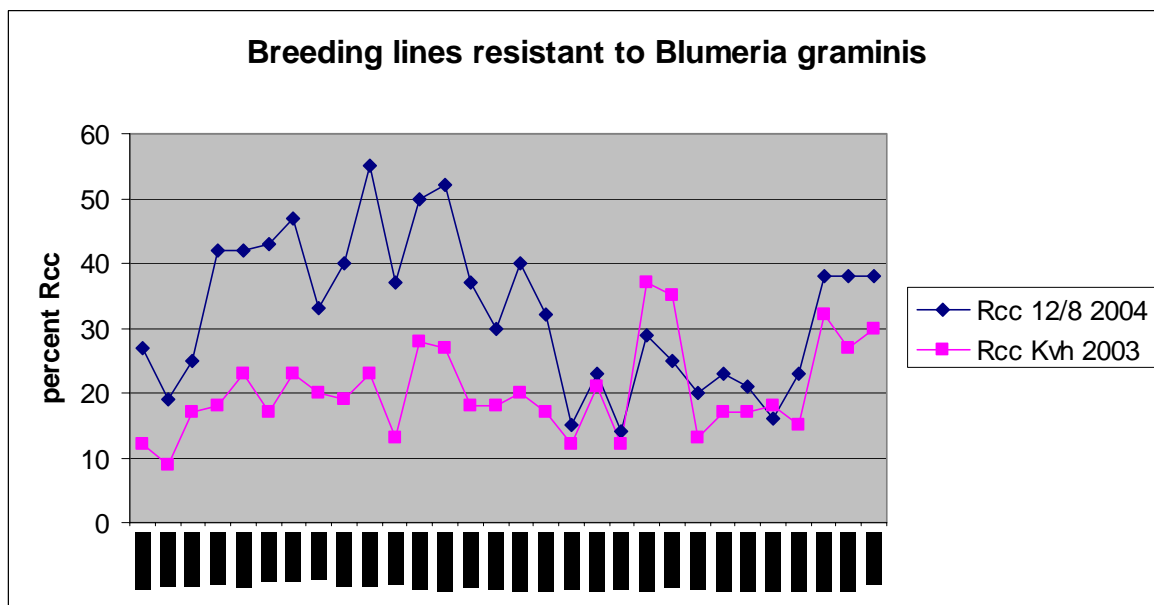
Figure 10. *Ramularia collo-cygni* severity scores in Graminor breeding *Blumeria graminis* resistant material. (Two years)

Figure 11. *Ramularia collo-cygni* severity scores in Graminor breeding *Blumeria graminis* resistant material (data for three years).

Some conclusions

We have screened several thousands of barley varieties, lines and breeding material for *R. collo-cygni* resistance during the period of years under investigation, <u>no complete resistance have been found.</u> This could be explained by a new way of understanding the *R. collo-cygni* phenomenon. (See Salamati & Reitan in the proceedings of this workshop).

Still there is space for increasing 'tolerance' to the fungus, i.e. reduce yield loss by heavy infection. We have experienced that tolerant varieties respond less to fungicide treatments, than non-tolerant ones.

Proper assessment of the disease is difficult when other barley diseases are present. To avoid this problem in *RLSB* nurseries, soils uninfected with other diseases are important. When possible, selective use of fungicides (early in the growing season) against other barley pathogens would reduce the problem. There are indications that small mildew attacks give induced resistance (tolerance) to *R. collo-cygni*.

There are *R. collo-cygni* 'tolerance' sources in NGB gene bank material.

Acknowledgement

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Screening for leaf spot resistance - results and impact on practical breeding

Herbert Bistrich[1], Josef Breun[2], Georg Emmert[3], Andreas Fleck[1], Heidi Jaiser[3], Harald Kempe[2], Marc Lemmens[3]

1) *Saatzucht Donau GesmbH & Co KG, Saatzuchtstr. 11, A-2301 Groß Enzersdorf, Austria*
2) *Saatzucht Josef Breun GdbR, Amselweg 1, D-91074 Herzogenaurach, Germany*
3) *Pajbjergfonden / Pajbjerg A/S, Grindsnabevej 25, DK-8300 Odder, Denmark*
4) *Universität für Bodenkultur,Wien, Interuniversitäres Department für Agrarbiotechnologie, IFA Tulln, Konrad Lorenz Str.20, A-3430 Tulln*

Abstract: Within the scope of a EC funded project (CRAFT 1999-70866), spring barley genotypes were evaluated for resistance to a leaf spot complex caused by *Ramularia collo-cygni*, non parasitic leaf browning (=NBV), or other. A core set of 55 genotypes was tested in replicated field trials at four locations in 2002 and at three locations in 2003. In addition 850 advanced breeding lines (screening set) and more than 1200 genebank accessions (pre-screening set) were tested in two and one environments respectively. Evaluation was done using a percentage damaged leaf area for the core set and a 1-9 scale for the other genotypes. To combine the core set evaluation data from time series (3 evaluation dates) into one value a linear disease progress was assumed. The area under disease progress curve (AUDPC) was calculated for each plot and expressed in % of the worst possible value. Overall variance analysis revealed significant genotype, environment and genotype x environment effects. Across all environments a quantitative variation of means was observed. *IPZ 24727* siblings showed the best resistance in both seasons whereas cv. *Barke* was found to be highly susceptible. A common feature of the worst cultivars (cvs. *Extract, Aspen, Avilla*) was possession of the mlo gene. These results corroborate the findings of Behn-Günther (2003) for NBV-QTL mapping. No released cultivar in the core set was surpassed the resistant check cv. *Prolog*. Cvs. *Jacinta* and *Millena* might be valuable crossing parents and several new additional resistance sources were identified. According to multiple comparisons of AUDPC means *SZD 159, SZD 160* and *Br 6680d36* were not significantly different from *LBPB 24727B*. In comparison with cv. *Prolog* lines with good resistance were frequently showing up in the screening set (e.g. *SZD 042-02, Br 104-02, Br 134-02, SZD 020-03, SZD 069-03, SZD 086-03, PF 016-03, PF 036-03*, PF 037-03) but promising lines were rarely found among the genebank accessions. The evaluation of those exotic lines was generally hampered by the high susceptibility to powdery mildew and leaf rust. Due to the severe drought in 2003, resulting in a lack of disease symptoms at all, a large part of the genebank material (trials at Herzogenaurach & Morgenrot) could not be evaluated. Nevertheless, cvs. *Chevron, Vairoga Priekuli, Emel Dschemal, Oberthal 7, Alpina, Irba Moda, Karez* and *Clermoni*, selected from the pre-screening set 2002, were showing good resistance at Lambach, Lipprichshausen and Großaitingen. For the core part, the correlations (spearmans ρ) between leaf spot susceptibility (AUDPC) and plant height or heading date were only weakly negative or not significant. The discovery of additional resistance sources combined with high operative heritabilities (0.8-0.9) and the given variation within ripening and plant height classes, should enable practical breeders to make a substantial progress in leaf spot resistance without a shift to late and tall genotypes. Resistance tests in controlled environments or techniques to ensure an even disease pressure (e.g. some kind of artificial inoculation) are not yet available.

Hence the selection of suitable testing sites, providing reliable disease development without the interference of other factors, will remain an important challenge. Last but not least, the breeder has to be on the site at the right time (to do the scoring).

Expression of resistance of barley varieties to Ramularia leaf spot and the status of the disease in Denmark

Hans O. Pinnschmidt [1] **and Susanne A. Sindberg** [2]

[1] *Danish Institute of Agricultural Sciences, Department of Crop Protection, Research Centre Flakkebjerg, DK-4200 Slagelse;* [2] *Danish Institute of Agricultural Sciences, Department of Variety Testing, Teglværksvej 10, Tystofte, DK-4230 Skælskør. Hans.Pinnschmidt@agrsci.dk*

Abstract: Ramularia leaf spot (RLS) has reached high severity levels in many spring- and winter barley cultivars in recent years at several sites in Denmark but the importance of the disease for Danish barley production is still unclear. Data from multi-environment trials indicated large varietal and environmental differences in RLS severity. Of the variation in RLS severity observed on individual barley cultivars, about 71% and 66% could be explained for spring- and winter barley, respectively, based on effects of the cultivar and the environment. The spring barley cultivars consistently exhibiting lowest levels of RLS severity were Power, Isotta, Cruiser and some others while Lonni, Lomerit, Nobilia, Chess and Carola were the winter barleys generally least affected by the disease. Such cultivars may be promising RLS resistance donors. Genetically closely related cultivars exhibited similar pattern with respect to statistical parameters describing their RLS reaction. The results suggest that varietal resistance can be an efficient mean for controlling RLS. The apparently most RLS-susceptible spring barley cultivars were those possessing mlo-resistance against powdery mildew while the apparently most RLS-resistant ones such as Power, Isotta and Cruiser do not. More work is needed to determine the nature and genetic basis of RLS resistance and to develop reliable methodology for efficient resistance testing.

Key words: *Ramularia collo*-cygni, resistance level, resistance stability, genotype x environment interactions, pedigree, Mlo

Introduction

Widespread and heavy infection of Ramularia leaf spot (RLS) was for the first time observed in Denmark in 2002 (Pinnschmidt & Hovmøller, 2003). Since then, survey activities and disease management-related research efforts such as investigations into resistance properties of Danish barley cultivars have been initiated. Observations of Burke *et al*. (2001), Cromey *et al*. (2002), Greif (2004) and Pinnschmidt & Hovmøller (2003, 2004) suggest that genetic variability in RLS resistance exists and can be used in resistance breeding and disease management. However, the importance of RLS for Danish barley production and options for RLS control via varietal resistance remain unclear. The objectives of this paper are to characterise the expression of RLS resistance of barley cultivars in view of potential uses for disease control and to provide an overview of the occurrence and severity levels of RLS in Denmark during the past years.

Material and methods

The major part of data presented here is from multi-environment surveys of the Danish Institute of Agricultural Sciences (DIAS), Variety Testing Division, in which commercial barley varieties and advanced breeding lines were grown in 1.2 x 10-15 m plots on more than 20 sites throughout Denmark from 2002 to 2005 (Anon. 2004, Anon. 2005, http://www.planteinfo.dk). One data set was obtained from a field trial conducted at the DIAS research centre in Flakkebjerg in 2003. In this trial, spring barley cultivars were inoculated with a suspension of RLS mycelium produced *in vitro*. The severity of RLS and other major leaf diseases (powdery mildew, net blotch, leaf rust, and leaf scald) was visually assessed as % diseased leaf area on each variety in each environment after the beginning of grain filling.

Only data sets from environments with at least 10% RLS severity on the most susceptible cultivar were considered for further analyses. RLS observations for a particular cultivar in a particular environment were only considered for analyses if the severity levels of any other foliar disease was $\leq$ 5% and the severity level of physiological leaf spots $\leq$ 10% on the respective cultivar in the respective environment. From the cultivars remaining after having applied these selection criteria, only those were included in further analyses that had valid observations for at least 2 years and 4 year-by-site environments.

General linear modelling (GLM) and Joint Regression (JR) analyses (e. g. Crossa, 1990) were employed to describe the data by additive main effects of the genotype (cultivar) and the environment as well as by genotype-specific and genotype-non-specific multiplicative effects of the mean RLS severity level of the environment. Parameters derived from these analyses, such as environment-adjusted mean RLS severity, standard deviation of environment-adjusted RLS severity, genotype main effect, environmental sensitivity slopes of genotypes, standard deviation of the residuals as well as projected RLS severity levels were used to characterise major features in the RLS reaction pattern of individual cultivars.

Results and discussion

RLS severity levels reached almost 40% in susceptible spring barley cultivars at Flakkebjerg in 2002 (environment 'Fla_02', Tab. 1). There were reports about high RLS severity levels at other sites in 2002, in spring- as well as in winter barley, but is was not possible to collect the corresponding data. The inoculated trial conducted in 2003 at Flakkebjerg (environment 'Fla_03') yielded only low RLS severity levels, as 2003 was perhaps not a conducive year for RLS development on spring barley. Neither were RLS attacks on spring barley reported from other sites in 2003. However, substantial RLS severity levels were again observed on spring barley at four sites in 2004 and at 10 sites in 2005, reaching up to 50% in some cases. The most severe RLS attacks were reported on winter barley (Tab. 2). On this crop, severe RLS severity levels were observed at several sites in 2003 and 2005, but not in 2004. Based on these observations, it can be concluded that Ramularia leaf spot is present in Denmark and has the potential to reach high severity levels that may be quite damaging. However, the occurrence of the disease in Denmark has hitherto been patchy in time and space. The overall significance of the disease for Danish barley production remains therefore unclear.

JR analyses indicated significant variety-specific effects of the environmental RLS severity level on the actual RLS severity of individual winter barley cultivars, beside highly significant variety-non-specific effects of the environmental RLS severity level (results not shown). Additive main effects of the variety were not significant in winter barley. In spring

barley, highly significant variety-non-specific effects of the environmental RLS severity level were indicated beside highly significant additive main effects of the variety. Variety-specific effects of the environmental RLS severity level were not significant. Of the total variation in RLS severity presented in Tab. 1 and Tab. 2, about 71% could thus be accounted for in spring barley and 66% in winter barley, respectively.

The spring barley cultivars exhibiting lowest levels of RLS severity in a relatively stable manner were Power, Nathalie, Isabella, Helium, Isotta, Cruiser and Modena, although a considerable number of observations was only available for Isotta, Cruiser and Power (Tab. 1). Nathalie and Isabella moreover displayed a similar RLS reaction pattern, as indicated by various statistical parameters (such as genotype main effect of RLS severity, mean environment-adjusted RLS severity, standard deviation of environment-adjusted RLS severity and standard deviation of residual RLS severity; results not shown), as their parent Power. The highest RLS severity levels amongst all spring barley cultivars were observed on Braemar, Smilla, Cabaret and Quench. The sister varieties Smilla and Cabaret moreover exhibited a similar RLS reaction pattern, as indicated by the statistical parameters mentioned above, that was resembling the one of their parent Dialog. Strikingly, the seemingly most RLS-susceptible spring barley cultivars (i. e. Braemar, Smilla, Cabaret and Quench, followed by others) possess Mlo-resistance against powdery mildew while the most resistant ones (i. e. Power, Isotta, Cruiser, etc.) don't (Anon. 2004, 2005).

The winter barley cultivar Lonni was almost completely free of RLS under all circumstances while Lomerit, Nobilia, Chess and Carola had low levels of RLS severity in a relatively stable manner (Tab. 2). The RLS reaction pattern indicated by statistical parameters for Carola was similar to the one of Carola's daughter Lonni (results not shown). The highest RLS severity levels amongst all winter barley cultivars were observed on Vanessa, Rafiki, 9013, Hamu, Celtic, Annerose and Escape.

Our results strongly suggest that efficient varietal resistance against RLS exists and, if enhanced by resistance breeding efforts, could play a vital role in achieving RLS control. Especially interesting resistance donor candidates may be the spring barleys Isotta, Cruiser, Power and others that may possess incomplete RLS resistance. Among the winter barleys, Lonni may be an interesting candidate for complete resistance while Lomerit, Nobilia, Chess and Carola may be interesting candidates for incomplete resistance. However, the true nature of resistance types, whether these are complete or incomplete, is yet to be revealed by in-depth studies and the genetics of RLS resistance and possible links to resistance properties related to other diseases, such as Mlo against powdery mildew, are yet to be disentangled. It will be essential for targeted RLS resistance breeding efforts to identify specific virulences and pathotypes in RLS populations and the matching resistance genes in barley genotypes. Developing robust methods for inoculum production and artificial inoculation and establishing a set of differential varieties will be crucial in this context.

Table 1. Mean Ramularia leaf spot severity (in %) after beginning of grain filling on spring barley varieties in multiple site-by-year environments. Note: for genotypes that have not been named officially, either the Danish numerical registration code or the breeders code is shown; cultivar 'Blanding 1079' is a mixture of Cicero, Simba and Smilla; environment codes are composed of the first 3 letters of the location and the last two digits of the year; location codes: Ask=Askov, Boe=Boelshoj, Brø=Brønderslev, Dur=Durup, Dyn=Dyngby, Fla=Flakkebjerg, Gri=Grindsted, Kar=Karise, Kol=Koldkærgård, Nr.=Nr. Åby, Ref=Refsvindinge.

variety	Ask_04	Ask_05	Boe_05	Brø_05	Dur_05	Dyn_04	Dyn_05	Fla_02	Fla_03	Gri_05	Kar_05	Kol_04	Kol_05	Nr._05	Ref_04	Ref_05
9003	3.0	0.1	0.5	0.0	.	18.0	5.0	13.8	.	0.0	0.1	0.5	5.0	8.0	18.0	0.0
9015	.	.	.	.	.	5.0	.	.	.	.	.	0.0	.	0.1	18.0	.
11807 A	1.0	.	1.0	.	10.0	25.0	25.0	.	.	.	0.1	10.0	.	3.0	8.0	.
Mauritia	18.0	1.0	3.0	3.0	.	33.0	18.0	.	.	.	3.0	10.0	25.0	1.0	33.0	0.0
Alexis	.	.	.	5.0	0.1	25.0	.	37.5	.	8.0	3.0	3.0	.	5.0	25.0	.
Alliot	18.0	.	1.0	.	5.0	50.0	25.0	31.3	12.5	8.0	0.5	5.0	5.0	0.5	25.0	3.0
Amalfi	3.0	5.0	3.0	3.0	1.0	25.0	33.0	.	.	18.0	3.0	10.0	0.0	18.0	50.0	3.0
Astoria	.	.	.	.	.	.	.	.	9.2	.	.	5.0	.	0.0	33.0	.
Aviator	5.0	0.0	0.0	.	1.0	18.0	10.0	.	.	.	0.1	3.0	1.0	3.0	33.0	0.0
Azalea	0.0	0.0	0.0	3.0	0.0	.	8.0	.	.	.	0.0	5.0	.	5.0	10.0	0.0
Barabas	1.0	5.0	0.5	3.0	3.0	10.0	18.0	.	.	3.0	0.0	3.0	5.0	3.0	8.0	0.0
Barke	0.1	1.0	3.0	.	.	10.0	18.0	10.0	.	10.0	0.0	1.0	10.0	3.0	18.0	.
Beatrix	0.0	.	0.0	.	0.0	5.0	8.0	.	.	.	0.0	18.0	0.0	1.0	3.0	.
Blanding 1079	10.0	8.0	5.0	3.0	5.0	33.0	33.0	.	.	10.0	0.5	1.0	10.0	3.0	50.0	1.0
Blanik	0.0	.	0.5	0.0	5.0	25.0	25.0	.	.	1.0	0.0	3.0	3.0	1.0	18.0	0.0
Br.7144b31	5.0	0.1	1.0	.	0.5	33.0	8.0	.	.	1.0	0.0	18.0	5.0	5.0	10.0	1.0
Braemar	25.0	8.0	5.0	.	5.0	33.0	33.0	25.0	.	25.0	8.0	8.0	5.0	1.0	50.0	3.0
Brazil	.	.	0.5	.	.	.	25.0	17.5	9.2	.	1.0	3.0	.	0.1	.	.
Cabaret	18.0	8.0	1.0	18.0	18.0	50.0	33.0	25.0	.	25.0	3.0	8.0	1.0	3.0	33.0	3.0
Carafe	8.0	0.1	5.0	.	.	8.0	25.0	.	.	5.0	0.5	8.0	10.0	5.0	33.0	0.0

Table 1. continued (1)

variety	Environment															
	Ask_04	Ask_05	Boe_05	Brø_05	Dur_05	Dyn_04	Dyn_05	Fla_02	Fla_03	Gri_05	Kar_05	Kol_04	Kol_05	Nr._05	Ref_04	Ref_05
Christina	10.0	.	0.0	.	.	.	10.0	.	.	.	0.0	3.0	10.0	0.0	25.0	.
Cicero	3.0	0.0	0.5	3.0	0.0	33.0	10.0	5.5	.	8.0	0.1	1.0	0.0	0.0	33.0	0.0
Class	5.0	1.0	0.5	.	0.0	5.0	25.0	11.3	.	.	0.0	3.0	3.0	0.1	8.0	0.0
Cruiser	0.5	1.0	0.0	0.0	0.0	5.0	10.0	7.5	.	.	0.0	3.0	0.0	0.0	10.0	0.0
Dialog	5.0	.	.	.	.	5.0	.	37.5	.	.	.	18.0	.	.	25.0	.
Edwina	10.0	8.0	3.0	10.0	8.0	.	33.0	.	.	5.0	0.0	8.0	5.0	3.0	18.0	3.0
Felicitas	8.0	.	0.0	.	0.0	10.0	10.0	25.0	.	.	0.5	1.0	3.0	0.1	10.0	.
Frieda	0.5	3.0	0.0	0.5	0.0	3.0	10.0	.	.	.	0.0	3.0	8.0	0.5	5.0	1.0
Frontier	.	.	0.0	.	.	.	8.0	.	.	.	.	3.0	.	0.0	33.0	.
Gizmo	3.0	.	0.0	3.0	0.0	18.0	18.0	.	.	.	0.1	8.0	.	1.0	10.0	.
Global	18.0	.	0.0	0.5	.	.	18.0	7.5	10.2	.	0.0	8.0	.	0.0	18.0	.
Hairoon	8.0	3.0	8.0	3.0	0.0	25.0	33.0	31.3	.	10.0	3.0	5.0	3.0	5.0	18.0	1.0
Hatifa	.	.	5.0	5.0	10.0	10.0	25.0	.	.	5.0	3.0	8.0	8.0	8.0	33.0	0.0
Helium	.	.	0.0	.	.	.	.	7.5	.	.	0.0	1.0	.	0.0	5.0	.
Henley	0.0	.	.	.	0.0	10.0	1.0	.	.	.	0.0	1.0	.	3.0	18.0	.
Hydrogen	8.0	8.0	3.0	3.0	0.0	18.0	18.0	17.5	5.0	.	3.0	1.0	.	0.0	18.0	.
Imidis	0.0	0.1	1.0	.	1.0	5.0	33.0	.	.	.	0.0	5.0	1.0	3.0	3.0	.
Isabella	.	.	0.0	.	.	.	8.0	.	.	.	0.0	1.0	.	0.0	3.0	.
Isotta	0.1	0.5	0.5	.	0.0	5.0	8.0	5.0	.	1.0	0.0	1.0	0.0	0.5	8.0	0.0
Jersey	5.0	.	.	.	.	8.0	.	17.5	.	.	.	1.0	.	.	10.0	.
Josefin	5.0	.	.	.	.	5.0	.	21.3	.	.	.	1.0	.	.	18.0	.
Justina	.	.	0.1	.	.	18.0	18.0	15.0	.	.	.	5.0	.	0.0	10.0	.
Kashmir	1.0	0.0	1.0	1.0	8.0	18.0	33.0	.	.	10.0	0.0	8.0	8.0	3.0	18.0	0.0
Katarina	1.0	3.0	1.0	8.0	1.0	33.0	25.0	.	.	.	0.1	5.0	.	1.0	10.0	.

Table 1. continued (2)

variety	Ask_04	Ask_05	Boe_05	Brø_05	Dur_05	Dyn_04	Dyn_05	Fla_02	Fla_03	Gri_05	Kar_05	Kol_04	Kol_05	Nr._05	Ref_04	Ref_05
							Environment									
Keops	0.5	1.0	8.0	18.0	5.0	10.0	50.0	.	.	.	0.0	8.0	.	1.0	18.0	.
Kulstof	1.0	5.0	3.0	5.0	5.0	.	25.0	.	.	.	0.0	3.0	8.0	8.0	10.0	.
Landora	5.0	0.0	0.5	0.0	0.0	8.0	18.0	17.5	.	1.0	0.5	3.0	1.0	0.0	10.0	.
Margret	3.0	0.0	.	1.0	.	18.0	.	.	.	0.0	.	1.0	.	3.0	33.0	.
Marigold	1.0	5.0	3.0	1.0	1.0	8.0	18.0	.	.	3.0	0.5	1.0	0.0	8.0	1.0	.
Marnie	5.0	.	.	.	.	3.0	.	13.8	.	.	.	3.0	.	.	8.0	.
Matinee	8.0	.	.	.	.	.	.	10.0	.	.	.	1.0	.	.	33.0	.
Mauritia	18.0	1.0	3.0	3.0	.	33.0	18.0	.	.	.	3.0	10.0	25.0	1.0	33.0	0.0
Mimer	0.0	.	8.0	.	1.0	10.0	33.0	.	.	0.0	0.1	1.0	0.0	1.0	8.0	0.0
Modena	1.0	.	.	.	.	.	.	1.0	1.7	.	.	0.5	.	.	10.0	.
Musikant	3.0	0.0	3.0	3.0	10.0	33.0	33.0	.	.	10.0	0.5	10.0	.	3.0	50.0	3.0
Nabiki	.	0.1	0.1	1.0	0.0	25.0	10.0	.	.	.	0.0	8.0	0.5	8.0	8.0	0.0
Nathalie	.	0.0	0.1	.	0.0	.	5.0	.	.	.	0.0	3.0	0.0	0.0	3.0	0.0
NFC Tipple	5.0	3.0	1.0	.	3.0	.	18.0	21.3	.	3.0	3.0	3.0	1.0	10.0	10.0	10.0
Otira	18.0	5.0	3.0	8.0	1.0	10.0	33.0	33.8	15.8	.	0.5	3.0	.	1.0	18.0	.
Parade	5.0	3.0	3.0	10.0	5.0	10.0	25.0	.	.	5.0	0.0	18.0	10.0	5.0	18.0	1.0
PF 19020-51	1.0	0.1	1.0	0.0	0.1	1.0	10.0	.	.	0.0	0.0	5.0	0.0	1.0	3.0	0.0
Picnic	0.5	3.0	3.0	.	1.0	8.0	10.0	.	.	0.5	0.1	1.0	5.0	0.5	3.0	0.0
Poet	1.0	5.0	3.0	.	5.0	5.0	18.0	.	.	.	0.5	3.0	0.5	3.0	8.0	0.0
Power	.	0.0	0.0	0.0	0.0	.	3.0	7.5	.	.	0.0	1.0	1.0	0.0	1.0	.
Prestige	33.0	5.0	3.0	5.0	.	8.0	25.0	17.5	.	10.0	8.0	10.0	18.0	0.5	25.0	.
Proctor	1.0	.	.	0.0	.	.	.	2.0	9.2	.	.	.	.	0.0	.	.
Publican	.	3.0	1.0	5.0	1.0	25.0	.	.	.	.	.	5.0	.	3.0	5.0	.
Quench	3.0	5.0	5.0	33.0	.	18.0	50.0	.	.	.	0.0	18.0	.	8.0	25.0	.

Table 1. continued (3)

variety	Ask_04	Ask_05	Boe_05	Brø_05	Dur_05	Dyn_04	Dyn_05	Fla_02	Fla_03	Gri_05	Kar_05	Kol_04	Kol_05	Nr._05	Ref_04	Ref_05
Scandium	0.0	0.5	0.1	0.1	1.0	3.0	8.0	.	.	3.0	0.5	0.5	0.1	5.0	0.5	1.0
Sebastian	25.0	.	.	.	0.0	.	.	17.5	.	.	.	5.0	0.0	0.0	25.0	.
Simba	18.0	.	3.0	8.0	8.0	25.0	33.0	17.5	.	10.0	5.0	3.0	5.0	0.0	25.0	8.0
Smilla	5.0	18.0	3.0	25.0	3.0	33.0	50.0	31.3	.	.	10.0	5.0	5.0	5.0	50.0	5.0
SW Immer	5.0	1.0	1.0	0.5	0.5	10.0	25.0	30.0	.	.	1.0	10.0	1.0	0.1	25.0	0.0
Texter	1.0	.	.	.	.	18.0	.	37.5	.	.	.	5.0	.	.	10.0	.
Tocada	.	.	0.0	.	.	25.0	.	.	.	.	0.1	5.0	.	3.0	18.0	.
Troon	10.0	.	5.0	.	10.0	25.0	25.0	25.0	.	.	3.0	3.0	3.0	3.0	25.0	5.0
Vanadium	1.0	5.0	3.0	1.0	0.0	18.0	33.0	.	.	3.0	0.0	8.0	8.0	5.0	18.0	1.0
Varberg	3.0	0.0	1.0	0.0	0.0	.	10.0	.	.	.	0.0	3.0	.	1.0	18.0	.
Westminster	1.0	0.5	1.0	0.0	0.0	8.0	10.0	.	.	3.0	0.5	3.0	3.0	10.0	5.0	0.0

Table 2. Mean Ramularia leaf spot severity (in %) after beginning of grain filling on winter barley varieties in multiple site-by-year environments. Note: for genotypes that have not been named officially, the Danish numerical registration code is shown; environment codes are composed of the first 3 letters of the location and the last two digits of the year; location codes: Abe=Abed, Bor=Borris, Dyn=Dyngby, Hob=Hobro, Jyd=Jyderup, Kar=Karise, Kol=Koldkærgård, Nr.=Nr. Åby, Ref=Refsvindinge, Sej=Sejet, Tys=Tystofte.

variety	Environment																
	Abe_03	Bor_03	Dyn_05	Hob_05	Jyd_03	Jyd_05	Kar_03	Kar_05	Kol_03	Nr._03	Nr._05	Ref_03	Ref_05	Sej_03	Sej_05	Tys_03	Tys_05
9009	.	1.0	5.0	10.0	.	1.0	1.0	.	.	33.0	10.0	1.0	0.5	5.0	1.0	20.0	10.0
9013	.	5.0	.	.	.	3.0	.	.	.	25.0	.	10.0	.	15.0	.	25.0	.
Annerose	0.0	0.0	25.0	18.0	5.0	1.0	1.0	.	0.0	33.0	33.0	5.0	10.0	5.0	8.0	25.0	18.0
Campanile	0.0	1.0	5.0	3.0	20.0	3.0	3.0	.	.	5.0	50.0	5.0	5.0	0.1	10.0	25.0	8.0
Carola	1.0	1.0	.	0.5	5.0	8.0	1.0	0.0	.	5.0	0.5	1.0	0.0	0.1	0.0	1.0	1.0
Celtic	5.0	5.0	.	8.0	25.0	1.0	8.0	1.0	0.0	8.0	.	15.0	0.5	5.0	0.1	100.0	8.0
Chess	0.0	0.0	10.0	0.5	0.0	0.5	0.0	0.5	0.0	0.0	.	0.0	0.1	0.0	5.0	0.5	3.0
Dolly	5.0	0.0	3.0	0.1	1.0	.	0.0	.	.	10.0	.	0.1	0.5	1.0	3.0	5.0	5.0
Escape	0.0	5.0	.	1.0	15.0	18.0	0.1	5.0	8.0	25.0	25.0	5.0	.	3.0	.	.	.
Hamu	.	3.0	.	.	25.0	8.0	3.0	.	.	1.0	.	10.0	3.0	1.0	5.0	50.0	.
Himalaya	5.0	0.5	8.0	8.0	10.0	10.0	0.0	1.0	0.0	5.0	25.0	0.0	1.0	1.0	8.0	10.0	10.0
Lomerit	0.0	0.0	.	.	5.0	.	0.0	3.0	0.0	0.5	.	1.0	0.0	0.0	0.5	0.5	0.1
Lonni	0.0	0.0	0.1	0.1	0.0	1.0	0.0	0.0	0.0	0.0	0.5	0.0	0.0	0.0	0.5	0.5	0.1
Ludo	3.0	0.0	.	.	.	.	0.5	.	.	.	.	5.0	.	.	0.5	20.0	.
Mombasa	.	0.0	25.0	.	8.0	.	3.0	.	.	20.0	.	0.0	.	5.0	5.0	15.0	.
Nobilia	1.0	0.0	0.5	0.1	5.0	3.0	0.0	.	1.0	3.0	.	0.1	0.0	0.5	0.0	3.0	0.1
Petrella	1.0	0.0	5.0	0.1	1.0	0.5	0.5	.	0.0	8.0	.	0.5	3.0	0.5	5.0	10.0	.
Rafiki	5.0	8.0	.	8.0	.	.	5.0	.	.	15.0	.	5.0	.	8.0	3.0	25.0	.
Regina	0.0	0.0	.	10.0	.	.	0.1	.	.	0.5	.	1.0	0.0	0.5	3.0	8.0	3.0
Vanessa	0.0	1.0	18.0	8.0	.	.	3.0	.	.	33.0	.	8.0	.	10.0	8.0	20.0	.

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Does the *mlo* resistance gene increase the susceptibility of spring barley to spotting diseases?

Joanne Claire Makepeace[1], James K.M. Brown[1], Simon Oxley[2] and James I. Burke[3]
[1] *Department of Disease and Stress Biology, John Innes Centre, Norwich Research Park,Colney, Norwich. NR4 7UH. UK.*
[2] *Scottish Agricultural College, Kings Buildings, West Mains Road, Edinburgh, Scotland.*
[3] *Teagasc, Oak Park, Carlow, Ireland.*
Correspondence: joanne.makepeace@bbsrc.ac.uk

Abstract: The mutant form of the *mlo* gene has been used in plant breeding as it confers durable resistance to *Blumeria graminis* (powdery mildew). However, the mutant gene carries undesirable pleiotrophic effects including spontaneous necrotic flecking of leaves which leads to reduced yield. Recent work has also shown that plants carrying a mutant *mlo* gene are more susceptible to two necrotrophic pathogens, *Magnaporthe grisea* (Jarosch et al., 1999) and *Cochliobolus sativus* (Kumar et al., 2001).

The aim of this project is to test if plants carrying mutant *mlo* alleles are more susceptible to three faculatative pathogens which are important in barley producing areas of northern Europe. These are *Rhynchosporium secalis*, *Pyrenophora teres* and *Ramularia collo-cygni*.

In field trials, near-isogenic lines of cvs. Ingrid and Pallas with mutant *mlo* alleles were more resistant than their recurrent parents to *R. secalis and R. collo-cygni*. This contrasts with the published results on *M. grisea* and *C. sativus*.

Growth room studies on seedlings showed that the environmental conditions affected the resistance of lines with mutant *mlo* alleles to *R. secalis*. The resistance of *mlo* mutant lines was associated with increased levels of host cell wall appositions (HCWA) produced by plant cells. These HCWA were associated with a reduction in penetration by the fungus. However, if the environmental conditions in which the plants are growing were altered prior to inoculation, a reduction or loss of the resistance was achieved. High light conditions prior to inoculation resulted in reduced resistance to *R. secalis* in *mlo* plants, associated with an increase in penetration and a reduction in HCWA produced by plant cells. A combination of high light and temperature prior to inoculation resulted in increased susceptibility of mutant *mlo* lines to *R. secalis*.

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Breeding for resistance against non-parasitic leaf spots in barley

Markus Herz, Anita Behn Lorenz Hartl, Günther Schweizer, Max Baumer
Bavarian State Research Centre for Agriculture, Institute for Crop Science and Plant Breeding, Am Gereuth 8 D-85452 Freising, Germany.

Abstract: Non parasitic leaf spots (NPLS) are a phenomenon which causes severe losses in yield and decrease in quality of barley. Especially in regions which are affected by intensive global radiation the symptoms can be observed frequently every year. Intensive radiation induces a stress response of the barley leaves which results first in brown spots and leads in later stages of the disease to a too early ripening of the plant. Often the lesions caused by the stress reaction are the entrance for a secondary fungal infection, which include in particular *R. collo cygni*.
On a particular location in Bavaria the symptoms of the leaf spots can be very well studied.
Genetic diversity of the response to global radiation was identified within the German barley germplasm. One breeding line showed extreme good resistance. The genomic localisation of the factors responsible for resistance could be identified by a QTL mapping approach. The knowledge about chromosomal assignment and linked markers was used to develop nearly isogenic lines carrying QTL intervals for resistance against NPLS.
The developed differential plant material will be the basis for further studies assessing directly the expression of the involved genes. Also the interaction of RCC with NPLS can be illuminated on the basis of the genetic background.
For the introgression of the resistance into adapted breeding material, efficient use of molecular markers will be an essential tool. The presented results describe the path to develop useful molecular markers for an important agronomic character of barley.

Genetics of spots and blotches in spring barley

Adrian C Newton and William TB Thomas
Scottish Crop Research Institute, Invergowrie, Dundee DD2 5DA, UK

Abstract: Random inbred lines from two contrasting spring barley crosses were used to study the genetics of Physiological Spotting (PS) and Ramularia Like Spots and blotches (RLS). The genetic control of PS was much higher than that of RLS and there appeared to be little association between the development of the two. Molecular marker maps of the two crosses were then used to search the genome for Quantitative Trait Loci affecting the expression of the two characters. Results showed that the *mlo* mildew resistance had a major effect upon the increased expression of PS. Apart from the *sdw1* dwarfing gene, no large genetic effects were detected in either cross for RLS, suggesting that deployable resistance was due to the cumulative action of minor genes in the crosses studied.

Chemical Control

Impact of fungicides and varietal resistance on *Ramularia collo-cygni* in spring barley

Simon Oxley[1], Neil Havis[1], Tony Hunter [2], Richard Hackett[3]

[1] *SAC, West Mains Road, Edinburgh EH9 3JG, United Kingdom.*
[2] *Biomathematics & Statistics Scotland, James Clerk Maxwell Building, The King's Building, Edinburgh, EH9 3JZ.*
[3] *Teagasc, Oak Park, Carlow, Eire.*

Abstract: *Ramularia collo-cygni* (Ramularia) has become a major disease of barley in Scotland and Eire. Growers currently rely on fungicides to achieve effective control, and this has led to an increase in production costs at a time when grain prices are low. The efficacy and yield benefits of fungicides to control Ramularia have been tested in Scotland and Eire in a three-year programme of 'Appropriate Fungicide Dose' trials. Prothioconazole and chlorothalonil remain the most effective options, but Quinone outside Inhibitor (QoI) fungicides are now ineffective. There are differences in the susceptibility of spring barley varieties to Ramularia. The UK variety Decanter has consistently shown good some resistance. Optic is intermediate, whilst the most susceptible varieties Pewter and Chariot are no longer UK commerical varieties. Most UK varieties are not severely affected by abiotic leaf spots, with the exception of Prestige. The most popular UK variety Optic can lose up to 0.5 t/ha yield as a consequence of Ramularia plus a decrease in grain quality. Yield losses from the more resistant varieties was lower at 0.3 t/ha.

Key words: Ramularia, barley, varieties, diseases, fungicides

Introduction

Ramularia collo cygni (Ramularia) has become a major barley disease in the UK and Eire. It became a major economic disease in 1998 (Oxley *et al.,* 2002), when may crops of the variety Chariot died back prematurely, leading to losses in yield and quality. The disease has been present every year since 1998, but the severity of the disease depends upon the variety and the weather conditions between tillering and ear emergence. Dull wet weather during this period leads to a higher incidence of the disease.

Current control measures rely on foliar fungicides applied at booting growth stage, before leaf spots appear. Not all fungicides are effective, and some, including fenpropimorph can be detrimental, leading to rapid loss in green leaf (Oxley *et al.,* 2002). A series of trials was undertaken to determine the efficacy and dose response of current and recently introduced fungicides. Longer-term solutions will rely upon varietal resistance. Yield and quality remain high priorities for plant breeders. Before investing in a programme for Ramularia resistance, breeders require more information that Ramularia is a disease worth investment. Information was also missing on the potential loss in yield and quality associated with Ramularia.

Material and methods

Appropriate fungicide doses

Over a three year period from 2003-2005, a total of five spring barley trials were sown to study the efficacy of fungicides on Ramularia. Sites and cultivars for the experiments were selected to maximise the severity of the target (Table1)

Table 1. Sites for fungicide efficacy trials

Year	Region	Variety	Target disease	Treatment Growth Stage (GS)	Spray date
2003	Carlow, Eire	Pewter	Ramularia	39-45	14 Jun 03
2004	Midlothian, Scotland	Pewter	Ramularia	43-49	16 Jun 04
2004	Carlow, Eire	Pewter	Ramularia	45-51	16 Jun 04
2005	Midlothian, Scotland	Prestige	Ramularia	57-59	22 Jun 05
2005	Carlow, Eire	Pewter	Ramularia	60-61	28 Jun 05

Each test fungicide (Table 2) was evaluated at a single timing at four doses 0.25, 0.50, 1.00 and 2.00 times the manufacturer's full recommended dose rate specified for barley to enable a dose-response curve to be fitted. Treatments were replicated three times, in randomised complete blocks.

Table 2. Fungicides and doses used in field trials

Active ingredient	Product	Full dose rate of product l/ha (g a.i.)
Untreated	---	---
Epoxiconazole	Opus®	1.00 l/ha (125)
Picoxystrobin	Acanto®	1.00 l/ha (250)
Pyraclostrobin	Vivid®	1.00 l/ha(250)
Prothioconazole	Proline®	0.80 l/ha (100)
Prothioconazole + fluoxastrobin	Fandango®	1.25 l/ha (125:125)
Azoxystrobin	Amistar®	1.00 l/ha (250)
Chlorothalonil	Bravo 500®	2.0 l/ha(1000)
Boscalid + epoxiconazole	Tracker®	1.5 l/ha (350:100)

Foliar diseases and percentage green leaf area were assessed visually on 10 tillers per plot approximately 3 and 6 weeks after application, to show the maximum extent of disease development and the best estimate of fungicide performance on each of the upper leaves. In some instances further assessments were done.

All trials were harvested and yielded. Grain moisture, specific weight and screenings (<2.5mm sieve) were determined. The statistical methodology adopted in other appropriate dose research (Paveley, 2000; Wale, 2000, Oxley & Hunter, 2005) was used. Disease and

green leaf data were transformed for the over trials analysis using a logit that allowed for the possibility of 0% or 100% in the data.

For each fungicide there were 4 data points at fungicide levels 0.25, 0.50, 1.00 and 2.00 and also the common untreated control point at fungicide level 0.00 i.e. 5 data points. For the purposes of exposition, previous studies have found that it was desirable that all curves pass through the untreated control point.

Varietal resistance to Ramularia

Ramularia, abiotic leaf spots and green leaf area were assessed on spring barley Recommended List (RL) trials. This series of work, funded by The Home-Grown Cereals Authority (HGCA), is used to provide data on existing and new varieties for the UK market. Average disease and green leaf scores were taken from the top two leaves in untreated plots in July, when crops were at milky ripe growth stages (GS73-77). A total of 8 trials were assessed over the three years.

Green leaf, Ramularia and abiotic leaf spots were were analysed using analysis of variance. Genstat (7th edition for Windows).

Yield loss

Yield loss trials were carried out at two sites in Scotland in each of four years (2002-2005). The varieties tested included Cellar, Chalice, Chariot, Optic, Pewter, Poker, Prestige, Spire, Troon and Westminster. Results have been reported for Chariot (susceptible to Ramularia and abiotioc leaf spots), Prestige (susceptible to abiotic leaf spots), Pewter (susceptible to Ramularia), Optic (intermediate for Ramularia) and Poker (less susceptible to Ramularia).

Not all varieties were present in each of the years. Fungicide treatments were applied as specified in table 3. The good and poor timings for Ramularia control were based on previous research (Oxley *et al*., 2002)

Table 3. Treatment timings for yield loss trials

Treatment	GS25-30	GS37	GS49
Untreated	Nil	Nil	Nil
No late treatment	Yes	Nil	Nil
Well timed second treatment	Yes	Nil	Yes
Poorly timed second treatment	Yes	Yes	Nil

Yes: Fungicide Applied, Nil: no fungicide applied.

The late fungicide treatment at GS49 is an important time to protect barley from Ramularia. The earlier treatment at GS37 was included in this trial series, since it provides the same amount of fungicide to the crop, but potentially leads to higher levels of leaf spots.

In 2003-204 the fungicides used at GS37 and GS49 were trifloxystrobin (Twist®) 1.0 l/ha + epoxiconazole (Opus®) 0.4 l/ha. In 2005, the fungicides used at GS37 and GS49 were prothioconazole (Proline®) 0.4l/ha + chlorothalonil (Bravo®) 1.0 l/ha + azoxystrobin (Amistar®) 0.5 l/ha.

Disease and green leaf area were assessed on the top three leaves in July at milky ripe growth stages (GS73-77). Scores for Ramularia and abiotic leaf spots were combined to provide an overall leaf spot score. Green leaf, Ramularia and abiotic leaf spots were analysed using analysis of variance. Genstat (7th edition for Windows).

Results and discussion

Appropriate fungicide doses

The QoI fungicides were the least effective at controlling Ramularia (Figure 1). In earlier research (Oxley *et al* 2002) good control was achieved with this group of fungicides. This suggests a major shift in resistance since 1999.

Fungicides which achieved effective control of Ramularia include chlorothalonil and prothioconazole. Epoxiconazole provides moderate levels of control, whilst the co-formulated fungicide epoxiconazole + boscalid achieved better control of Ramularia than epoxiconazole alone.
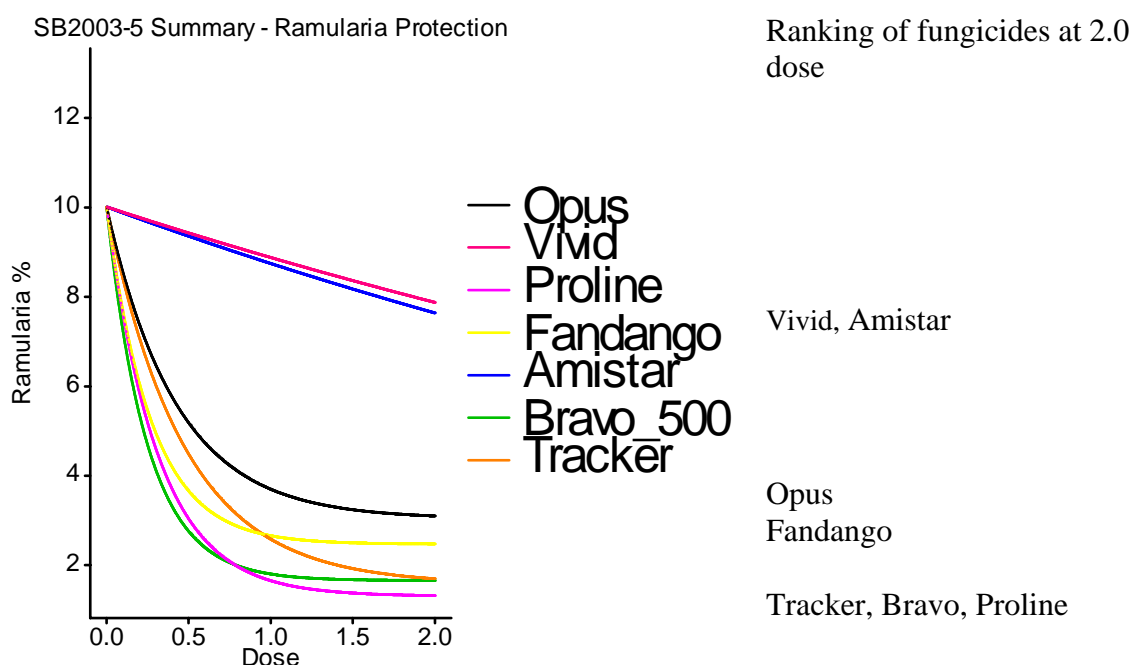
Figure 1. Ramularia control on the top leaves

Green leaf area retention showed a similar pattern, chlorothlaonil and prothioconazole achieving the best green leaf area retention (Figure 2). Note the greening effect from QoI fungicides when applied alone was poor.
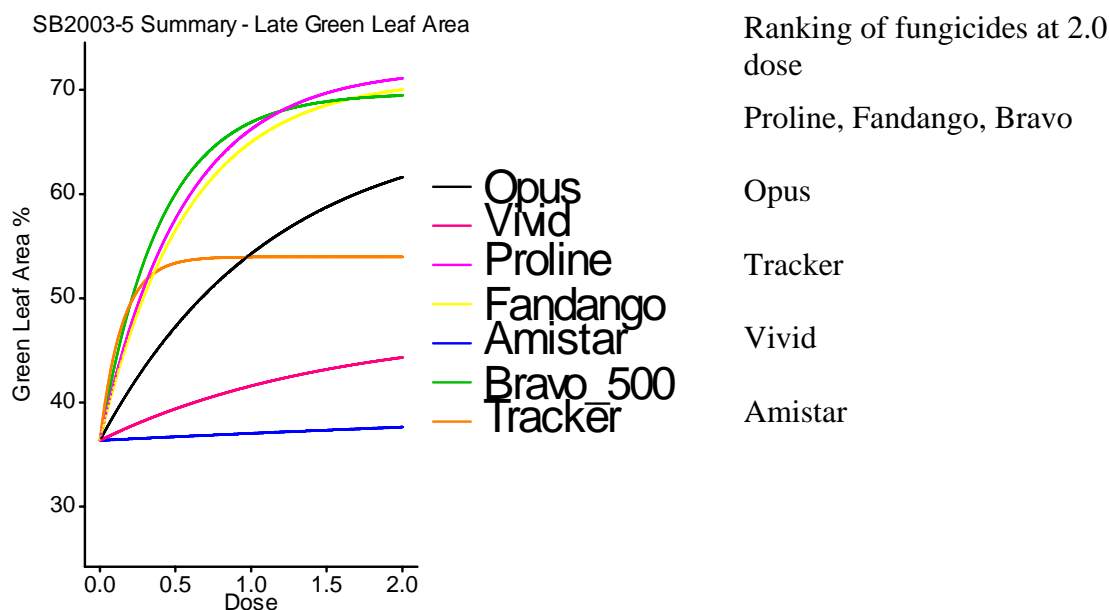
Figure 2. Green leaf area retention on the top leaves

Best current practice in Scotland to achieve effective control of Ramularia is to apply prothioconazole and chlorothlaonil. Although QoI fungicides (e.g. azoxystrobin) achieved little impact when applied alone, they continue to contribute to yield, when applied in a mixture with the other groups of fungicides.

Varietal resistance to Ramularia

Table 4 shows the average levels of Ramularia, abiotic leaf spots and green leaf area retention on the upper leaves for a range of spring barley varieties currently on the Recommended List in the UK.

Differences between the varieties in Ramularia, abiotic leaf spots and green leaf are significant. Some varieties have only been in trial for one year, but most of the varieties have been in trial for three years. Most UK spring barley varieties now have low levels of abiotic leaf spots. The variety Prestige (which was removed from the list for 2006), is a variety affected most by abiotic leaf spots, with an average level of over 10%.

No variety shows complete resistance to Ramularia, but the variety Decanter is the only one with levels less than 10%. The variety Cocktail at the other end of the spectrum shows the highest levels of Ramularia averaging 20%.

Green leaf area scores can be misleading, since later maturing varieties would be expected to have higher levels of green leaf area than early maturing varieties area on any given date. The most susceptible varieties Cocktail and Prestige were amongst the varieties with lower green leaf scores, whilst Decanter and Westminster which had lower levels of Ramularia had higher green leaf area scores. This suggests the most susceptible varieties do senesce early as a result of high levels of Ramularia.

Table 4. Varietal resistance to Ramularia and abiotic leaf spots.

Variety	% Ramularia	% Abiotic leaf spots	% green leaf area	Number of years in trial
Decanter	9.1	2.3	55.5	3
Power	10.6	2.1	42.8	2
Appaloosa	11.6	2.9	46.1	1
Riviera	12.4	3.5	39.8	3
Poker	12.5	2.4	49.8	1
Static	12.5	4.6	42.9	3
Waggon	13.3	2.7	40.7	2
Westminster	13.3	1.9	51.0	2
Oxbridge	13.8	2.6	42.7	2
Tocada	14.1	2.2	36.8	2
Spire	14.2	3.8	40.3	3
Wicket	14.6	2.2	36.6	1
NFC Tipple	15.4	0.8	44.1	2
Kirsty	16.7	2.4	31.8	3
Optic	16.7	2.9	40.3	3
Cellar	17.7	3.1	32.3	3
Troon	18.0	3.0	36.0	3
Chalice	18.1	3.1	35.2	3
Doyen	18.9	2.3	35.5	3
Prestige	19.3	11.4	31.5	3
Rebecca	19.3	2.2	31.5	3
Cocktail	20.4	5.6	35.0	3
Sed.	2.22	2.12	4.78	
LSD	4.53	4.32	9.73	
Df.	32	32	32	
Sig.	<.001	<.001	<.001	

Yield loss

Yields for the fungicide treatments are presented in Table 5. Comparing the untreated yield with the well timed late treatment is not the best method to determine the yield loss due solely to Ramularia, since *Blumeria graminis* (powdery milew) and *Rhynchosporium secalis* (Rhynchosporium) are two other key diseases which affect spring barley in Scotland. A comparison of the ″no late treatment″ with the ″well timed late treatment″ gives a better measure of the yield loss from Ramularia leaf spots. The ″well timed″and ″poorly timed″ treatments did not always give good separation of good and poor control of leaf spots with the same fungicides applied.

Yield losses due to Ramularia and abiotic leaf spots for each variety were Pewter (0.45 t/ha), Chariot (0.37 t/ha), Optic (0.53 t/ha) Prestige (0.32 t/ha) and Poker (0.27 t/ha).

Table 5. Yields (T/ha) for spring barley varieties in response to a range of fungicide programmes.

Variety	Untreated	No late treatment	Well timed late treatment	Poorly timed late treatment	Comment on Ramularia resistance
Poker	6.48	6.81	7.08	6.80	Good
Optic	5.97	6.35	6.88	6.83	Intermediate
Pewter	6.42	6.42	6.87	6.79	Poor
Prestige	6.17	6.08	6.40	6.28	Poor (abiotic)
Chariot	6.06	6.08	6.45	6.14	Poor (biotic & abiotic)
		Fungicide	Variety	Fungicide x variety	
	Sed.	0.102	0.161	0.395	
	Lsd.	0.200	0.316	0.775	
	Df.	764	764	764	
	Sig.	<.001	<.001	Ns	

As grain prices fall, growers may question the need to take action with a fungicide. Grain quality is equally as important as yield, since the value of grain is based on grain meeting the market requirements. For malting barley, screenings (% of grain which falls through a 2.5 mm sieve) is a useful measure of quality for malting barley, and growers will receive penalties for the value of the grain where screenings are above 10%. Table 6 shows the screenings values for the five varieties.

A reduction in screenings by the well timed treatment was most obvious in Optic at 3.5% . Pewter and Chariot screenings were reduced by a similar amount albeit from a lower initial value.

Table 6. Screenings (<2.5 mm sieve) for spring barley varieties in response to a range of fungicide programmes.

	Untreated	No late treatment	Well timed late treatment	Poorly timed late treatment	Comment on Ramularia resistance
Poker	7.4	7.3	6.0	6.7	Good
Optic	20.8	16.7	13.2	13.9	Intermediate
Pewter	12.1	12.1	8.4	10.2	Poor
Prestige	8.3	7.4	4.5	4.4	Poor (abiotic)
Chariot	13.3	13.9	10.5	11.7	Poor (biotic & abiotic)
	Fungicide	Variety	Fungicide x variety		
Sed.	0.901	1.142	3.489		
Lsd.	1.769	2.796	6.850		
Df.	766	766	766		
Sig.	<.001	<.001	Ns		

Ramularia reduction with a well timed fungicide was more successful in the varieties with good or intermediate resistance. It was a greater challenge in varieties susceptible to abiotic leaf spots (Table 7).

Table 7. % Leaf spots (Ramularia and abiotic) in spring barley varieties in response to a range of fungicide programmes.

	Untreated	No late treatment	Well timed late treatment	Poorly timed late treatment	Comment on Ramularia resistance
Poker	16.8	13.3	4.0	3.3	Good
Optic	26.9	23.9	12.1	16.2	Intermediate
Pewter	23.4	21.7	13.5	21.0	Poor
Prestige	37.0	28.2	19.2	15.7	Poor (abiotic)
Chariot	29.4	27.9	22.6	25.6	Poor (biotic & abiotic)
	Fungicide	Variety	Fungicide x variety		
Sed.	1.68	2.66	6.51		
Lsd.	3.30	5.21	12.77		
Df.	766	766	766		
Sig.	<.01	<.001	Ns		

Green leaf area levels on the upper leaves were improved by treating crops at GS49 or GS37. For most varieties, the best green leaf area retention was seen with the GS49 application, but Prestige responded well to a GS37 application. Green leaf area retention was greatest in the more resistance variety Poker.

Table 8. % Green leaf area in spring barley varieties.

	Untreated	No late treatment	Well timed late treatment	Poorly timed late treatment	Comment on Ramularia resistance
Poker	36.7	40.8	76.7	73.3	Good
Optic	26.8	41.1	61.2	48.6	Intermediate
Pewter	28.4	30.7	50.8	39.3	Poor
Prestige	19.2	26.7	45.0	60.0	Poor (abiotic)
Chariot	22.6	25.2	39.4	28.5	Poor (biotic & abiotic)

	Fungicide	Variety	Fungicide x variety
Sed.	3.95	6.25	15.31
Lsd.	7.76	12.27	30.06
Df.	766	766	766
Sig.	3.95	6.25	15.31

Conclusions

Ramularia has become an established disease in Scotland, and it consistently causes yield losses of up to half a tonne per hectare and a loss in quality. Current varieties have good resistance to abiotic leaf spots, but most continue to be susceptible to Ramularia. Fungicides can currently provide effective control of leaf spots, but potential resistance to the QoI fungicides, and pressures on growers to cut costs mean that varietal resistance remains the main priority to manage this disease in the future.

Acknowledgements

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Control of Ramularia in winter barley and spring barley using different fungicides - Experience from Denmark

Lise Nistrup Jørgensen, Anne Christiansen

Danish Institute of Agricultural Sciences, Department of Integrated Pest Management, Research Centre Flakkebjerg, DK-4200 Slagelse. LiseN.Jorgensen@agrsci.dk

Abstract: In 2004 and 2005 different fungicides were tested for their efficacy for control of Ramularia leaf spot (*Ramularia collo-cygni)* in both spring barley and winter barley. The disease did not appear at any significant extent before GS 59-65. Ramularia leaf spot reached a level of 10-50% severity assessed on the 2-3 upper leaves at GS 75-77. In 2 trials in spring barley, fungicides were applied at GS 45-51. The fungicides Bell (boscalid + epoxiconazole), Opera (epoxiconazole + pyraclostrobin) and Proline (prothioconazole) gave between 87% and 96% control when 66% of the normal rate was used. Significant dose responses on efficacy were seen for 66% and 33% of the tested dosages. In 4 winter barley trials fungicides were applied at GS 39-51 comparing 50% of normal rates. The best effect was obtained by using Bell, Opus Team (epoxiconazole + fenpropimorph), Opera and Proline. In one winter barley trial 3 different timings for control of Ramularia leaf spot were compared. The results showed best control from application at either GS 37-39 or 51-55. The later timing (GS 61-65) gave significantly lower levels of control. In this trial Bell gave better control than Opera, which again was better than Acanto Prima (picoxystrobin + cyprodinil). In both spring and winter barley the yield responses from controlling Ramularia leaf spot (3-11 hkg/ha) were significant. The level of control of Ramularia leaf spot was only slightly reflected in the yield responses partly due to the fact that also other diseases appeared in the trials. The best margin over fungicide cost was obtained following the use of reduced rates (33% rate) of Opera and Bell.

Key words: fungicides, Ramularia leaf spot, winter barley, spring barley

Introduction

Ramularia leaf spot (*Ramularia collo-cygni*) was first observed in Denmark in 2002 (Pinnschmidt & Hovmøller, 2004). As in other countries the disease has often been confused with symptoms of abiotic necrotic spots (PLS). In 2004 PLS was seen more frequently in winter barley compared with Ramularia leaf spot, whereas in 2005 Ramularia leaf spot was found to dominate. Ramularia leaf spot has been found in both winter barley and spring barley. So far the disease has only appeared from GS 59-65, and there is so far no evidence of earlier symptoms being found in the fields.

Disease control in barley crops in Denmark is normally carried out using 1 treatment between GS 31 and 51, depending on the disease development. The experience has been that in winter barley one treatment around GS 37-39, using approximately 33-50% of a normal rate, gives the best margin over fungicide cost (Jørgensen, 2004; Landsforsøgene, 2005). In spring barley the experience has been that 0-25% of normal rate gives the best margin over

fungicide cost (Jørgensen et al., 2000; Hagelskjær et al., 2003). However, if early attacks of powdery mildew or net blotch occur, two treatments have been found profitable in very susceptible cultivars (Hagelskjær et al., 2003; Landsforsøgene, 2005). The objectives of this study were to investigate the potential loss from controlling Ramularia leaf spot as well as to investigate which fungicides, dosages and timings to recommend in order to get the best control.

Material and methods

Trials were conducted for two years (2004, 2005) testing the effect on Ramulaira leaf spot and yield parameters from different fungicides. The trials were carried out as randomised block trials with 4 replicates and a plot size of 15-25 m^2. The trials were carried out either at Flakkebjerg experimental station or at farmers' sites. Susceptible cultivars were chosen for these experiments. The spring barley trials were carried out in the cultivar Alliot and the winter barley trials in the cultivar Vanessa. The times of application in individual trials varied between GS 39 and 51. Specifically in one trial 3 different timings were compared.

Disease assessments reported in this paper were all made as percent leaf area covered by Ramularia leaf spot or other diseases present on a total green crop stand. Assessments were made at several growth stages. In the trials the dominant disease was in all cases Ramularia leaf spot.

In all trials spraying was carried out with a plot sprayer at low pressure (2-3 bar) using flat fan nozzles and a volume of water of 200 litres/ha. The different fungicides tested are listed in Table 1, which includes information on trade name, active ingredients and normal dose. The plots were harvested with a plot combine harvester. The dry matter content was measured, and the grain yield was corrected to 15% moisture content. Margin over fungicide cost was calculated in hkg/ha for the different treatments with the costs of fungicides and application being deducted.

Statistical analysis on the data was done by using ANOVA, from which LSDs were calculated at the 95% confidence levels.

Table 1. Fungicide products used in the trials.

Product	Normal rate	Active ingredients per litre
Acanto Prima	1.0 l/ha	80 g picoxystrobin + 300 g cyprodinil
Amistar	1.0 l/ha	250 g azoxystrobin
Bell	1.5 l/ha	233 g boscalid + 66.6 g epoxiconazole
Comet	1.0 l/ha	250 g pyraclostrobin
Opera	1.5 l/ha	133 g pyraclostrobin + 50 g epoxiconazole
Opus Team	1.5 l/ha	84 g epoxiconazole + 250 g fenpropimoprh
Opus	1.0 l/ha	125 g epoxiconazole
Proline	0.8 l/ha	250 g prothioconazole
Stereo	1.6 l/ha	62.5 g propiconazole + 250 g cyprodinil
Unix 75WG	1.0 kg/ha	750 g cyprodinil

Results and discussion

Spring barley

In the two field trials from 2004 and 2005 in spring barley, Ramularia leaf spot did not develop before GS 61-65. At GS 75 the level of attack reached 15-20% assessed on the 3 upper leaves. Minor attacks of net blotch (*Drechslera teres*) were present in the trials earlier on, but this disease did not exceed more than 2-6% in untreated (Table 2).

The fungicides Bell, Opera and Proline all gave good control of Ramularia leaf spot, and 4 weeks after application between 87% and 96% control was obtained using 66% of the normal rate. Significant dose responses were seen between 66% and 33% of the tested fungicides, the dose response was lowest for Bell. The residual effect of Bell was also better compared with Proline, Opera and Opus.

Yield responses in the 2 spring barley trials, which were dominated by Ramularia leaf spot, varied between 5 and 11 hkg/ha. The response related poorly to the products' efficacy on Ramularia leaf spot. Best margin oven fungicide cost was obtained following the use of 33% of the normal rate of Opera and Bell or the mixture Amistar + Stereo. The increase in yields from fungicide treatments was related to better thousand grain weight and improved screening.

Table 2. Per cent attack of net blotch and Ramularia leaf spot in spring barley following one application at GS 45-51 using different fungicides. 2 trials 2004 and 2005 (04344/05345).

Treatments	Dose/ha	% Net blotch		% Ramularia		Yield and yield increase hkg/ha		Margin hkg/ha	
	L/ha	2004	2005	2004	2005	2004	2005	2004	2005
Untreated		2.3 a	6.3 a	21.2 a	15.8 a	53.6	63.1	-	-
Amistar + Unix	0.33+ 0.33	0.1 d	0.1 d	11.0 b	7.3 b	9.2	7.5	4.4	2.7
Amistar + Stereo	0.33+ 0.53	0.1 d	0.1 d	7.2 c	6.0 bc	10.2	7.6	5.7	3.1
Opera	1.0	0.4 c	0.1 d	2.5 e	2.3 d	12.0	7.1	5.1	0.2
Opera	0.5	0.4 c	0.3 d	5.5 d	4.3 c	10.8	6.0	6.9	2.1
Proline	0.54	0.3 d	0.4 d	1.5 f	0.5 e	11.2	5.8	5.9	0.5
Proline	0.27	0.6 bc	1.8 b	3.2 e	2.3 d	7.3	5.3	4.2	2.2
Bell	1.0	0.2 d	0.2 d	1.4 f	0.2 e	10.8	7.4	4.9	1.5
Bell	0.5	0.2 d	0.2 d	2.7 e	0.3 e	9.4	7.3	6.0	2.1
Acanto Prima	1.0	0.1 d	-	7.1 c	-	10.2	-	5.1	-
Acanto Prima	0.5	0.2 d	-	11.6 b	-	7.4	-	4.4	-
Opus	0.66	-	0.9 c	-	2.3 d	-	5.3	-	0.6
No. of trials		1	1	1	1	1	1	1	1
Growth stage		77	74	77	75				
DAT		27	22	27	27				
LSD$_{95}$						1.4	5.2		

Winter barley

In 4 winter barley trials carried out in 2005, fungicides were applied at GS 39-51 testing 50% of normal fungicide solutions. Also in winter barley Ramularia leaf spot did not develop before GS 61-65. The attack in untreated varied between 10-50% attack assessed at GS 75. On average the best effect was obtained using Bell, Opus Team, Opera and Proline (Table 3,

Figure 1) Generally, the effects of triazoles were better than the effect of strobilurins (Comet, Amistar and Acanto).

In winter barley the yield responses following fungicide treatments were relatively low (3-8 hkg/ha). The level of control was only slightly reflected in the yield responses partly due to the fact that other diseases also appeared in the trials.

In the timing trial from 2005 3 different timings for control of Ramularia leaf spot were compared, spraying at GS 37-39, 51-55 and 61-65 respectively. At GS 75 the trial had 15% attack of Ramularia leaf spot, and the results showed best control from treatments at either GS 37-39 or 51-55 (Figure 2). The later timing (GS 61-65) gave significantly lower levels of control. In this trial Bell gave better control than Opera, which again was better than Acanto Prima. The yield responses from the 2 early timings were better than treatments carried out at GS 65. Although the effect from Acanto Prima was inferior to Bell and Opera, the yield responses did not differ significantly between the 3 fungicides. The impact of Ramularia leaf spot on photosynthetic activity was investigated further in this trial, and the results were reported by Christiansen and Wollenweber (2006).

Table 3. Comparison of 50% rates of different fungicide solutions used for control of leaf diseases in winter barley applied at GS 39-51, 4 trials 2005 (05335).

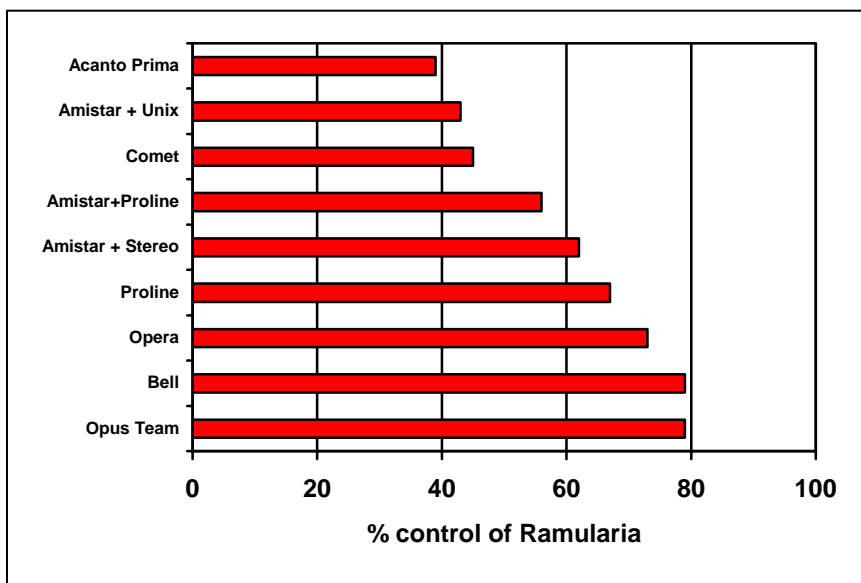| Treatments at GS 39-51 Dose/ha L/ha	% Net blotch		% Barley rust	% Ramularia		% Powdery mildew		Yield and yield increases hkg/ha	Margin hkg/ha
Untreated	2.5	a	8.3 a	24.5	a	14.3	a	74.2	
0.5 Comet	0.6	b	1.4 b	13.4	b	3.9	b	5.3	-0.1
0.75 Opera	0.8	b	2.0 b	6.5	c	3.8	b	6.4	1.0
0.75 Bell	0.6	b	1.0 b	5.2	c	3.3	b	6.1	1.5
0.25 Amistar + 0.4 Stereo	0.6	b	1.1 b	9.4	bc	5.2	b	5.7	2.1
0.25 Amistar + 0.25 Unix	0.6	b	1.0 b	13.9	b	3.8	b	5.4	1.5
0.25 Amistar + 0.2 Proline 0.4	0.6	b	1.4 b	10.5	b	4.7	b	5.1	1.0
Proline	0.7	b	1.5 b	8.1	bc	5.7	b	5.4	1.2
0.75 Acanto Prima	0.7	b	1.5 b	15.1	b	4.9	b	4.9	0.8
0.75 Opus Team	0.9	b	1.3 b	5.1	c	2.6	b	7.0	2.8
No. of trials	2		3	4		4		4	4
Growth stage	71-73		75-77	75-77	37-	75-77			
DAT	28-29		40-41	42		36-41			
LSD$_{95}$								2.2	

Figure 1. Control of Ramularia leaf spot in 4 winter barley trials using 50% of normal rate of different fungicide solutions applied at GS 39-51. Attack in untreated was approximately 25% at GS 75-77 when assessments were carried out.
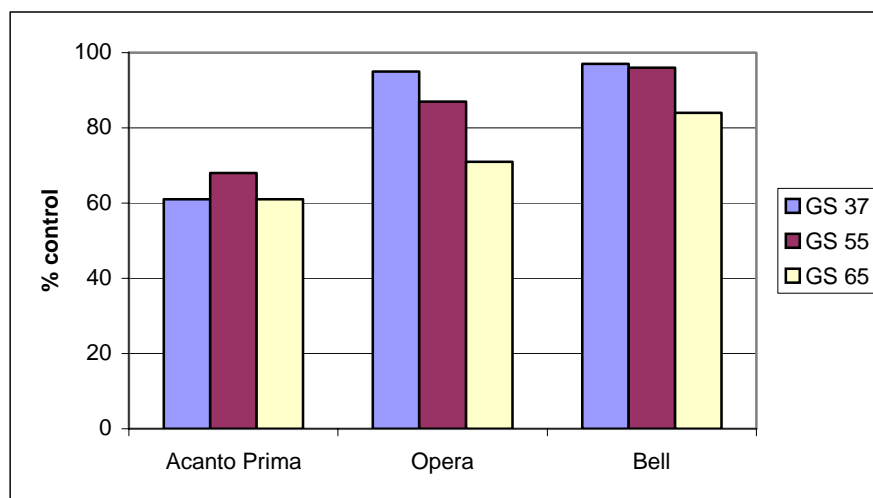

Figure 2. Control of Ramularia leaf spot in one winter barley trial using 50% of normal rate of 3 different fungicides applied at 3 different timings. Attack in untreated was 15% at GS 75.

General discussion of results

In other countries similar good control of Ramularia leaf spot have been found using Bell and Proline (Klingenhagen, 2006; Balz et al., 2006). Bell has in Danish trials also proved to give good control of symptoms, which have been categorised as PLS and which were widespread in 2004.

In the Danish results products with epoxiconazole have been having good effect on Ramularia leaf spot. The experience from using products containing epoxiconazole are slightly more variable abroad. The results from Germany (Klingenhagen, 2006; Balz et al., 2006) are not so convincing, whereas results from Ireland confirm good control from epoxiconazole products (Dunne, 2003).

Strobilurins have in some investigations been found to give good control (Burke et al., 2001; Harvey, 2002). In the Danish trials the strobilurins pyraclostrobin, picoxystrobin and azoxystrobin have given some control, but the effect has generally been inferior to other products. This has also been the experience from German trials (Klingenhagen, 2006).

Products containing chlorothalonil have given very good and persistent control in other countries (Balz et al., 2006; Dunne, 2003). At present it is not expected that this product will be authorised in Denmark.

The results from other countries also confirm that the optimal timing is just before or around the time of heading (Balz et al., 2006; Burke et al., 2001).

Conclusions

Ramularia leaf spot is in Denmark not recognised as a disease developing before GS 59-65. Several effective fungicides for control of Ramularia leaf spots have been identified, including epoxiconazole, prothioconazole and boscalid. Application between GS 37 and GS 55 has proved to give effective control. Yield responses from controlling Ramularia leaf spot have been cost effective but the yield losses have still been relatively limited in both spring and winter barley despite relatively severe attacks. Low-dose strategies using 33% of the normal recommended rates for control of Ramularia have proved to be effective solutions giving the best margin over fungicide cost.

Acknowledgements

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Chemical control and strategies against *Ramularia collo-cygni*

Torsten Balz[1], **Günter Prigge**[2], **Ulrich Krieg**[3], **Ulf Sattler**[4], **Andreas von Tiedemann**[1]

[1] *Department of Crop Sciences, Division of Plant Pathology and Crop Protection, University of Göttingen, Grisebachstr. 6, 37077 Göttingen;*
[2] *BASF Aktiengesellschaft; Agrarzentrum Limburger Hof, Carl-Bosch-Straße 64, 67117 Limburgerhof;*
[3] *Bayer Crop Science Deutschland GmbH; Elisabeth-Selbert-Straße 4a, 40764 Langenfeld;*
[4] *Syngenta Agro GmbH, Am Technologiepark 1-5, 63477 Maintal*

Abstract: Over the last few years *Ramularia collo-cygni* (Rcc) has been observed very late in the barley growing season. In 2004 we saw the first infections of Rcc at the end of May on plants at growth stage EC 69. On May 24th the causative agent was found on leaves 3 and 4 and one week later, on May 31st, it was found on the flag and first leaves. One explanation for this late infestation by Rcc is that older plants are more susceptible. Infection of the plants is initiated by spores and can be seen by studying wind borne spores and Rcc proteins in barley leaves. Examination of spores on water agar showed no pronounced optimum temperature for spore germination within a range of 4°C to 24°C. Fewer spores were seen to germinate above 24^oC and germination rate was even lower above 32°C.

At present none of the barley varieties available provide a useful tool for a strategy against Rcc. All varieties are susceptible and analysis showed no differences in the levels of Rcc protein found in the straw. The timing of initial infestation and the speed of disease development varies with variety.

In contrast to control through resistant varieties, fungicides have been found to provide a useful control strategy against Rcc. We applied selected fungicides at one application timing, growth stage EC 37-39, at the full recommended rate. The treatments were compared according to direct disease control and persistence of effect and yield. Opera (50 g/l epoxiconazole and 133 g/l pyraclostrobin at 1.5 l/ha) showed a moderate control of disease. Champion (233 g/l boscalid and 67 g/l epoxiconazole at 1.5 l/ha) was better than Opera. Input (160 g/l prothioconazole and 300 g/l spiroxamine at 1.25 l/ha) gave very good disease control but the persistence of effect was not so good with Rcc symptoms appearing 6 weeks after treatment and disease developing thereafter. Fandango (100 g/l prothioconazole and 100 g/l fluoxastrobin at 1,25 l/ha) seems like the same like Input, but the persistence effect stops earlier. The treatments with the best combination of direct disease control and persistence of effect were Bravo (500 g/l chlorothalonil at 2 l/ha) and Amistar Opti (400 g/l chlorothalonil and 80 g/l azoxystrobin at 2.5 l/ha). Yields of Opera, Champion, Fandango, Input, Amistar and Amistar Opti treatments are on a comparable level. The results will be presented and discussed in the lecture.

Fungicide impact on Ramularia Leaf Spot in barley as analysed by chlorophyll fluorescence imaging

Anne Christiansen[1], Bernd Wollenweber[2] and Lise Nistrup Jørgensen[1]
1) Department of Integrated Pest management. 2) Department of Genetics and Biotechnology
Danish Institute of Agricultural Sciences Research Centre Flakkebjerg, Forsøgsvej 1, DK-4200 Slagelse, Denmark.

Abstract: The impact of Ramularia leaf spot and fungicide applications on photo-synthesis was studied using chlorophyll imaging. In a factorial field trial either on of three fungicides (Acanto Prima, Opera and Bell) was applied in either one of three growth stages (GS 37-39, 51-55 or 61-65). Photosynthesis measured as chlorophyll fluorescence decreased with increasing disease levels in general whereas photosynthesis in green areas adjacent to necrosis remained unaffected. The efficiency of PS(II) measured as Fv/Fm was here decreased 34 % from healthy to infected leaves which has also previously been published for biotrophic fungi. With the synthesis of toxin, necrotrophic fungi have further been verified to cause even more severe reductions of Fv/Fm. *R. collo-cygni* synthesise phytotoxins (rubellins) apparently without causing the same reduction of Fv/Fm as has been published for necrotrophs. Visual assessments and measurement of chlorophyll fluorescence were performed on F-1 leaves (1st leaf under flag leaf) for three weeks at weekly intensity, starting after symptoms emergence in GS 65. Fungicides with epoxiconazole (Opera and Bell) gave significantly better control of disease than Acanto Prima. The two earliest timings (GS 37-39 and 51-55) gave also better control than the latest timing (GS 61-65). Assessments in week 25 revealed that all treatments except Acanto Prima applied at the latest growth stage gave significantly better control of disease compared to untreated. No significant differences were found in the treatment strategies effect on photosynthesis measured as chlorophyll fluorescence. However Acanto Prima had a significantly higher ETR than the other fungicides even though it did not control RLS significantly. Assessments of ETR made in week 24 were significantly higher than in week 23 and 25 respectively. No differences in the efficiency of PS(II) (Fv/Fm) could be found, except that assessments made in week 24 were significantly different from week 23 and 25.

Key words: Ramularia leaf spot (RLS), chlorophyll fluorescence, apparent rate of photosynthesis (PS), quantum yield (Fv/Fm), light response curves, fungicides, application timings.

Introduction

Ramularia leaf spot (RLS) was recognised in Denmark in 2004 as an increasing problem in barley production (Pinnschmidt & Hovmøller, 2004). This fungal disease, which has been known from other European countries for several years, can cause yield losses about 30 % (Cromey *et al.*, 2002, Pinnschmidt & Hovmøller, 2004). Fungicide applications along with less susceptible cultivars are a potential way to control RLS. Apparently no cultivar is able to avoid RLS completely and research in chemical strategies can be a useful tool in disease control. Fungicides can have a curative or preventive effect on disease. Which way is the right might be dependent on the pathogen emergence and ecology. To date only a little is known about the epidemiology of *R. collo-cygni* and when and during which conditions it is

pathogenic. It is well known that the RLS which are following the leaf veins mainly on leaves exposed to light and awns are caused by synthesis of rubellins during infection (Heiser *et al.,* 2004). Furthermore these photodynamic active rubellins cause oxidative stress in plants by inducing the formation of reactive oxygen species (ROS). ROS can cause lipid per-oxidation and co-oxidation of pigments, seen as necrotic spots with a yellow halo (Heiser *et al.*, 2003). Damage to the plant caused by ROS formation is prevented in plants by the activity of several anti-oxidative enzymes scavenging oxygen radicals. However sometimes antioxidants can be overwhelmed with necrotic symptoms as consequence (Scandalios, 1993). Further ROS can prevent pathogens by itself or activate further plant resistance genes (Buchanan *et al*., 2002).

Chemical control strategies are tools to keep leaves green and in turn prevent yield losses. For the control of RLS it might be important to apply fungicides before symptoms develop, which is still difficult to forecast. Chlorophyll fluorescence imaging is a non-invasive technique to visualize infections before damage appears and to quantify heterogeneous differences caused by biotic and abiotic stress (Chaerle, 2004).

The aim of this study was to follow the impact of RLS as well as the effect of fungicide applications to the photosynthesis by use of chlorophyll fluorescence imaging and visual assessments of disease.

Material and methods

Field trial
A factorial trial field trial with the winter barley cultivar Vanessa was located at the Danish Institute of Agricultural Sciences, Flakkebjerg in the growing season of 2004/2005. Either one of following fungicides: Acanto Prima, Opera or Bell was applied in either one of the following growth stages: 37-39 (week 19), 51-55 (week 21) or 61-65 (week 22), which gave an amount of 10 treatments (inclusive untreated) in 4 replicates. Table 1 shows the details of current fungicides.

Table 1. Product names and active ingredients of fungicides used in the field trial for control of Ramularia leaf spot.

Product name	Active ingredients per litre
Acanto Prima Opera Bell	80g Picoxystrobin + 300g Cyprodinil 133g Pyraclostrobin + 50g Epoxiconazol 233g Boscalid + 66.6g Epoxiconazol
Untreated	-

A rate of 0.75 litre per hectare was used for all treatments being equivalent to 50 % of the recommended dosage. Assessments of disease on 1st leaves under the flag leaf (F-1) were started when symptoms appeared in week 23 (after GS 65).

Chlorophyll fluorescence imaging
Leaves (F-1) in untreated plots were categorized according to a following 9 category scale: 0, 0-0.1, 0.1-1, 1-5, 5-10, 10-25, 25-50, 50-75 and 75-100 percent RLS. Chlorophyll fluorescence was measured for every category in order to verify the impact of increasing disease levels on photosynthesis.

All treatments in the trial were assessed for percent RLS on F-1 leaves along with measurement of chlorophyll fluorescence at the 3 samplings dates with weekly intervals (week 23, 24, 25), in order to verify the impact of fungicides on RLS and in turn photosynthesis. Twelve different F-1 leaves (11 infected and 1 healthy) were sampled from each treatment. Leaves were collected in clear plastic bags to keep them moist and immediately measured for chlorophyll fluorescence with a Imaging Pam Chlorophyll Fluorometer (Walz, Effeltrich, Germany).

Four leaves were measured at a time. Each leaf was cut 5 cm from the basis and placed with the adaxial side upwards and the cut ends against each other. Thirteen flashes of light (650 and 780 nm) with increasing intensities from *PPFD* (Photosynthetic Photon flux density) 1 - 461 μmol m^{-2}s^{-1} were applied by the Image PAM apparatus. The following parameters were measured in specified Areas Of Interest: Maximum PS (II) quantum yield (Fv/Fm) (=efficiency of PS(II)) and apparent rate of photosynthesis (PS) (=corresponding to the electron transport rate (ETR)). In addition, light response curves were analysed and calculated as electron transport rate (ETR) (= electron transport from PS(II) to PS(I)) over the increasing light intensities.

All data was analysed using the GLM procedure through pc-SAS (release 8.2; SAS Institute, Cary, NC, USA) P$\leq$ 0,05.

Results and discussion

Impact of RLS on photosynthesis as analysed by chlorophyll imaging
Images of 4 out of 9 disease categories with the apparent rate of photosynthesis measured in untreated plots with increasing infection levels are shown in Figure 1. This parameter corresponds mostly to the electron transport rate (ETR). Images in figures are displayed through high light intensity (*PPFD* 461μmol m^{-2}s^{-1}).

Inhibition of photosynthesis was only detected after the appearance of first necrotic symptoms. By following the colour bar at the bottom in the Figure 1, it is possible to follow that photosynthesis was affected in spots following the leaf veins indicated by colours others than pink on the images. Later, these spots merged in lengthy necrotic areas on leaves, seen as black areas on the images. The gradient from active to non-active areas is very small, indicating that photosynthesis in green areas adjacent to necrosis remained unaffected. Maximum fluorescence (PS-values) is 49.8 on all disease level which is normal for healthy leaves (Oxborough, 2004).

It was found that increasing disease level affected the photosynthesis in general. The effect of RLS on the fluorescence variable: quantum yield (Fv/Fm) at low light intensities (*PPFD* 1) is shown in Figure 2. The efficiency of PS(II) (Fv/Fm) had a linear relationship with disease severity with R^2 being 0.94. In symptom less areas Fv/Fm was about 0.75-0.80, which is normal for healthy leaves (Oxborough, 2004), whereas it was reduced about 34% with 75-100 % disease during low light intensities.
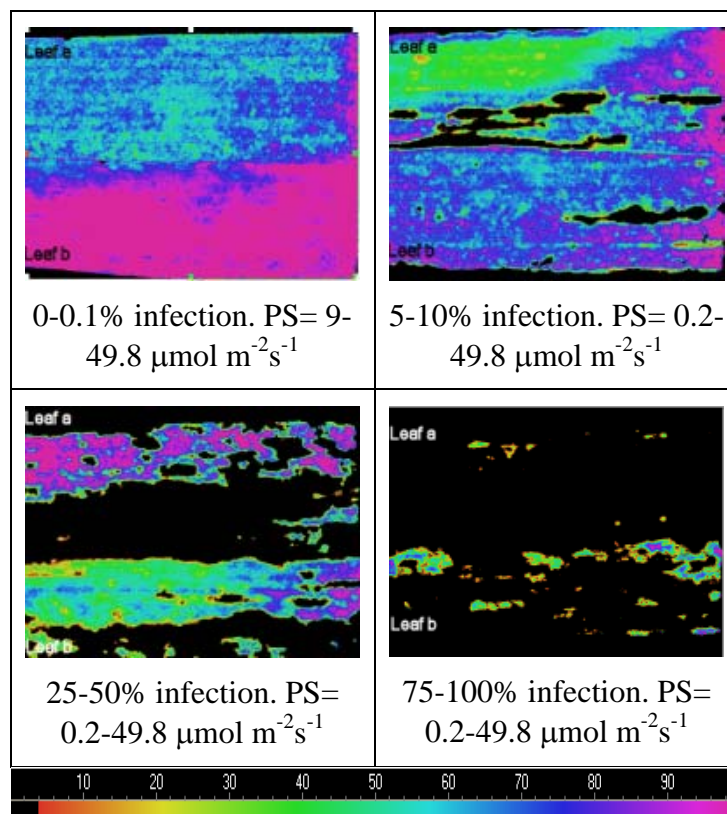
Figure 1. Apparent rate of photosynthesis during *PPFD* 461 with increasing level of RLS. The false colour bar at the bottom verifies the photosynthetic activity in the measured leaf area. Black areas indicates necrotic areas whereas pink are fully active areas. Min. and max. values are calculated for each image.
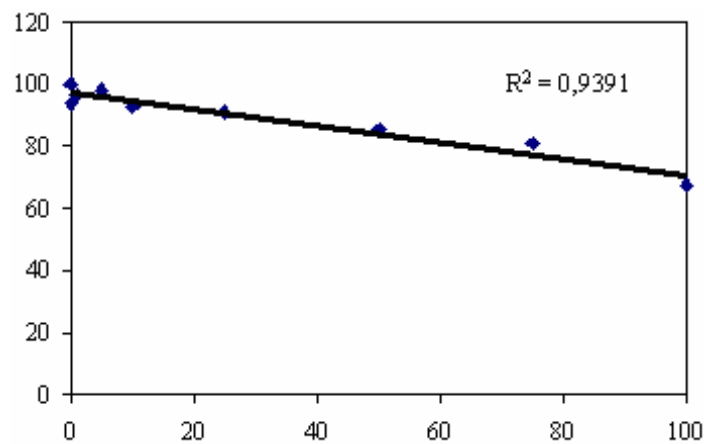

Figure 2. Percent quantum yield (Fv/Fm) as a function of percent RLS on F-1 leaves during low light intensities (*PPFD* 1).

Effect of fungicide application

Visual assessments of disease severity on F-1 leaves for all treatments from week 23-25 are shown in Table 2. In week 23, there were no significant differences between treated and untreated plots. In week 24, all treatments except application of Acanto Prima in GS 61-65 controlled disease significantly. In week 25, all treatments except Acanto Prima applied at the latest growth stage gave significantly better control of disease compared to untreated but there was a tendency of better control with Opera and Bell applied to all growth stages. Bell and Opera gave significantly better control of RLS than Acanto Prima when data was analysed as a factorial trial. There was also a general tendency to lower levels of control for all 3 fungicides from the latest application timing.

Table 2. Effect of fungicide application on percent RLS on F-1 leaves for the weeks 23-25.

GS	Fungicide	% RLS on F-1		
		Week 23	Week 24	Week 25
37-39	Acanto Prima	0,0 A	0,6 B	23,8 BC
	Opera	0,0 A	0,3 B	2,8 C
	Bell	0,1 A	0,0 B	0,6 C
51-55	Acanto Prima	0,0 A	1,1 B	22,0 BC
	Opera	0,3 A	0,9 B	7,8 BC
	Bell	0,4 A	0,3 B	2,4 C
61-65	Acanto Prima	0,2 A	2,3 AB	36,3 AB
	Opera	0,2 A	1,5 B	20,0 BC
	Bell	0,4 A	1,4 B	4,9 BC
	Untreated	0,2 A	5,3 A	61,3 A

Impact of fungicide application on photosynthesis as analysed by chlorophyll imaging

Figure 3 shows the quantum yield (Fv/Fm) and the light response curves measured as electron transport rate (ETR) over a range of increasing light intensities.

There was a significant difference in photosynthesis activity during the three weeks. Assessments of maximum ETR from week 24 were significantly higher than for week 23 followed by 25 which are also shown in figure 3. Application with fungicides kept the differences between healthy and infected leaves for a longer period of time compared to untreated (Figure 3).The three timings were not significantly different. The enhanced maximum ETR in infected leaves compared to healthy looking leaves is reflecting compensation in photosynthetic activity in the affected leaves. Apparently there were no significant differences between treatments but when testing the trial as a factorial trail, Acanto Prima had the significantly highest ETR followed by Opera and Bell. The fungicides Opera and Bell were not significantly different. Assessments of Fv/Fm in week 24 were significantly different from assessments in week 23 and 25. Maximum Fv/Fm was significantly lowered in infected compared to healthy leaves which is reflecting an increasing disease severity causing

less effective PS(II). No significantly differences could be found neither between treatments nor between fungicides or timings when data was analysed as a factorial trial.
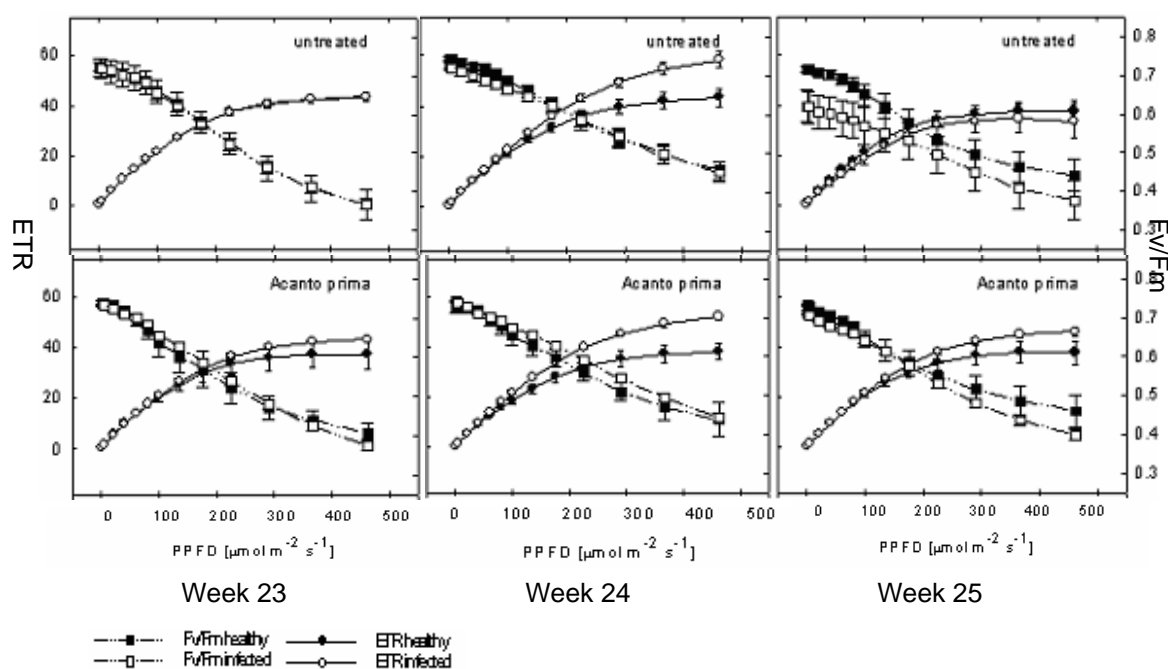

Figure 3. Light curves measured in ETR and quantum yield curves from untreated and Acanto Prima applied in GS 51-55. Results presented are from week 23-25.

Discussion

Ramularia leaf spot decreased photosynthesis in areas with symptoms but did not appear to do it in apparently healthy regions (Figure 1). Photosynthesis in the remaining tissue may compensate for the lost area and can even be stimulated for a while with induction of disease (Buchanan *et al.,* 2002). Trials made earlier with biotrophic, hemibiotrophic and necrotrophic fungi have revealed, that fungi can be distinguished from each other by the way they affect photosynthesis in areas with and without symptoms (Bassanezi *et al.*, 2002). Biotrophic fungi might not affect photosynthesis in green leaf areas to the same extent as hemibiotrophic and necrotrophic fungi. Necrotrophic fungi secreting enzymes or phytotoxic compounds which can diffuse to other regions of the leaf can cause direct closure of stomata or reduction of enzymes in the Calvin cycle which in turn change the concentration of soluble carbohydrates in infected tissues which in turn affect photosynthesis (Bassanezi *et al.* 2002). Rubellins might not have this kind of impact for this interaction. These differences in areas with and without symptoms have been verified with rust caused by the biotrophic fungus *Uromyces appendiculatus* in beans which reduced the quantum yield (Fv/Fm) about 35% compared to about 100 % with hemibiotrophic fungi like *Colletotrichum lindemuthianum* (Bassanezi *et al.,* 2002). Ramularia leaf spot caused here 34 % reduction of Fv/Fm. Even though *R. collo-cygni* synthesise phytotoxins (rubellins) and the infection follows the leaf veins, it does not appear to induce lowered photosynthesis in symptom free regions as would be expected from necrotrophs but apparently more like for biotrophs (Figure 2). The biology of *R. collo-cygni* is still poorly investigated but results here might indicate that the fungus partly act as a biotroph as the photosynthesis was apparently unaffected in green leaf areas and partly as a necrotroph

by synthesising rubellins which cause the necrotic spots without causing the same extent of lowered photosynthesis in green leaf areas.

The fungicides Opera and Bell with the active ingredient epoxiconazol were here responsible for disease control. Similar results have been obtained from other trials in Denmark while experience with this ingredient is more variable in other countries (Jørgensen & Christiansen 2006). Application of triazoles (epoxiconazol) and to a lesser extent strobilurins (kresoxim-methyl and azoxystrobin) has been found to increase plant defences and the chlorophyll synthesis as well the overall net-photosynthesis (Tiedemann & Wu 2001, Bertelsen *et al*. 2001). Here the fungicide Acanto Prima enhanced ETR at high light intensities in infected leaves to a higher degree and for a longer period of time than application of the other fungicides, even though it did not control RLS well.

During the measurement of chlorophyll fluorescence there can be few sources of error which might affect the results. During oxidative stress xanthophylls like zeaxanthin, are synthesised and might enhance light utilization in green leaves (Buchanan *et al*., 2002) but earlier studies on these issues have revealed this to be of minor importance (Peterson, 1995). Any decrease in the ability of the Calvin cycle to accept electrons via NADPH could also lead to an increase in fluorescence as the electron transport is slowed down. Further as leaves were collected in plastic bags and transported for the measuring of chlorophyll fluorescence, a following stomata closure could have led to less efficient utility of light. For the latter statement it can be concluded that all leaves were treated similarly and differences could still be found between healthy and infected leaves.

Conclusions

Chlorophyll imaging showed that photosynthesis was decreased with increasing disease levels and that photosynthesis in areas without symptoms remained more or less unaffected. The efficiency of PS(II), (Fv/Fm) was affected more like has earlier been published for biotrophs and less like hemibiotrophs and necrotrophs. As it was not possible to measure any damaging impact of disease on photosynthesis before symptoms emergence, the chlorophyll imaging might not be a useful tool to forecast disease. Instead it can be used to verify further issues of this fungus biology in leaves.

Visual scorings of Ramularia leaf spot revealed that all treatments except Acanto Prima applied at the latest growth stage gave significantly better control of disease compared to untreated but there was a tendency of better control with Opera and Bell applied to all growth stages. The best time of application was significantly best in GS 37-55. The use of chlorophyll imaging in verifying the effect of current fungicide application was not obvious. There could not be found any significant differences in the measurement of any of the treatments impact on photosynthesis, but Acanto Prima seemed to be able to enhance the ETR more than for the other fungicides even though it was not able to control disease effectively. The enhancement of the photosynthesis is a plant strategy to compensate for the lost area which in turn gain yield. There was also a significant difference seen between healthy and infected leaves for all treatments. The difference in photosynthesis between infected and healthy looking leaves was larger in the second assessment weeks than in the first followed by the third week. The use of chlorophyll fluorescence imaging was able to verify the effect of a fungicide application but not to verify any differences in application timings. Earlier this chlorophyll imaging technique has proved its potential by measuring transients of several kinds of stress factors impact on photosynthesis (Chaerle, 2004).

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Benefits of triazole + chlorothalonil for the control of *Ramularia collo-cygni*, a new problem in Barley in France

Laurent Dany
Syngenta Agro, France, 78210 St-Cyr-L'Ecole

Abstract: In 2002, *Ramularia collo-cygni* (R c-c) generated severe yield losses in the center of France. Since this year, the presence of this fungus has been also frequently notified in other areas of France. The symptoms of this disease are often considered as those of *Dreshlera teres*. Since 2004, a monitoring was made in various areas of France, isolations were made in Petri dishes and revealed the widespread presence of *R c-c*.

Some trials with exclusive detection of *R c-c* showed a very high level of efficacy of triazole + chlorothalonil based products (Bravo Premium or Citadelle). The results of these specialities are better than straight triazole applications.

Key words : *Ramularia collo-cygni*, chlorothalonil, Bravo Premium, Citadelle.

List of Participants

Last name	First name	Organ./Comp.	Address/ Phone/ E-mail
Andersen	Ole	Sejet Plantbreeding	Nørremarksvej 67, Sejet, 8700 Horsens, Denmark, +45-75 68 2177; OAN@SEJET.COM
Bachem	Ulrich	DuPont de Nemours (D) GmbH	Schulkoppel 6, D-23919 Rondeshagen, Germany, 04544-891219, Ulrich.Bachem@dupont.com
Backes	Gunter	The Royal Veterinary and Agricultural University, Department of Agricultural Sciences	Thorvaldsensvej 40, DK-1871 Frederiksberg C, Denmark, +45 3528 3434, guba@kvl.dk
Balz	Torsten	Syngenta Agro GmbH	Germany, +49 172 6698522, Torsten.Balz@syngenta.com
Berg	Gunilla	Swedish Board of Agriculture	Plant Protection Center Box 12, SE-230 53 Alnarp, Sweden, +46 40 41 52 96, gunilla.berg@sjv.se
Bistrich	Herbert	Saatzucht-Donau GesmbH Co KG	Reichersberg 86, A-4986 Reichersberg/Inn, Austria, +43 7758 4001-16, herbert.bistrich@saatzucht-donau.at
Boot	Kees	CEBECO-SEEDS B.V.	Lisdoddeweg 36, 8219 PR Lelystad, Netherlands, 31 320 225700, Cees.Boot@cebeco-seeds.com
Brown	James	John Innes Centre	Colney, Norwich, NR4 7UH, U.K.+44-1603-450615, jame.brown@bbsrc.ac.uk
Bruckmeier	Stefan	Saatzucht Ackermann	Ringstr. 17, 94342 Straßkirchen, Germany, +49 9424 942341, bruckmeier@sz-ackermann.de
Bury	Paul	Syngenta Seeds	Market Stainton, Market Rasen, Lincolnshire LN8 5LJ, U.K., +44 1507 343348, Paul.bury@syngenta.com
Caron	Daniel	ARVALIS Institut du Végétal	Station Inter-instituts, 6, Chemin de la Côte Vielle 31 450 BAZIEGE, France, +33 5 62 71 79 39, d.caron@arvalisinstitutduvegetal.fr
Christiansen	Anne	Danish Institute of Agricultural Sciences Research Centre Flakkebjerg, Dept. of Integrated Pest Management	ForsØgsvej 1, Flakkebjerg, 4200 Slagelse, Denmark, +45 8999 1900, Anne.Christiansen@agrsci.dk
Cordsen Nielsen	Ghita	Danish Agricultural Advisory Service, National Centre, Crop Production	Udkaersvej 15, DK-9200 Aarhus N, Denmark, +45-87405000 Direct: 0045-87405439, gcn@landscentret.dk
Czembor	Henryk J.	IHAR-Plant Breeding and Acclimatization Institute, Plant Breeding and Genetics Depmt.	IHAR-Radzikow, 05-870 Blonie, Poland, +0 0 22 796 33 73, h.czembor@ihar.edu.pl
Czembor	Jerzy H.	IHAR-Plant Breeding and Acclimatization Institute, Plant Breeding and Genetics Dept.	IHAR-Radzikow, 05-870 Blonie, Poland, +0 0 22 796 33 73, j.h.czembor@ihar.edu.pl

Last name	First name	Organ./Comp.	Address/ Phone/ E-mail
Dany	Laurent	Syngenta Agro	20, rue Marat, 78 210 Saint-Cyr-l'Ecole, France, +33 1 39 42 23 73, Laurent.dany@syngenta.com
Dietze	Thomas	Fr. STRUBE Saatzucht KG	Postfach 13 53, 38358 Schöningen, Germany, +49 5354-809 46, t.dietze@fr-strube.de
Djurle	Annika	Dept. of Mycology and Pathology, Swedish University of Agricultural Sciences	Box 7026, SE-750 07 Uppsala, Sweden, +46 18 671602, Annika.Djurle@mykopat.slu.se
Einfeldt	Claus	Saatzucht Ackermann	Ringstr. 17, 94342 Straßkirchen, Germany, 0049 9424 942341, Einfeldt@sz-ackermann.de
Eriksen	Birger	Sejet Plantbreeding	NØrremarksvej 67, DK-8700 Horsens, Denmark, +45 7568 2177, bee.@sejet.com
Forster	Birgit	Syngenta Crop Protection AG	Schwarzwaldallee 215, P.O. Box, CH-4002 Basel, Switzerland, +41 61 323 28 67, Birgit.forster@syngenta.com
Frei	Peter	Agroscope RAC Changins, Station Fédérale de Recherches Agronomiques	Case postale 10 12, CH-1260 Nyon 1, Switzerland, +41 22 363 43 77, peter.frei@rac.admin.ch
Godwin	Jeremy	Syngenta Crop Protection AG	Research Biology, Wst-540.1.73, CH-4332 Stein, Switzerland, +41 61 323 1171, jeremy.godwin@syngenta.com
Greif	Peter	Saatzucht Streng GmbH & CO KG Germany	Aspachhof, 97215 Uffenheim, Germany, +49 9848-97998-32, p.greif@aspachhof.de
Guicherit	Eric	Syngenta Crop Protection AG	Schwarzwaldallee 215, CH-4002 Basel, Switzerland, +41-61-323 5775, eric.guicherit@syngenta.com
Hagemeister	Heinz	Bayer Crop Science Germany	Elisabeth Selbert Strasse 4 a, 40764 Langenfeld, Germany, +49 2173–2076–289, heinz.hagemeister@bayercropscience.com
Hanusova	Martina	SELGEN Co.	Stupice, 25084 Sibřina, Czech Republic, +420281012451, hanusova@selgen.cz
Hasselfeld	Frank	Syngenta Seeds	Zum Knipkenbach, 32107 Bad Salzuflen, Germany, 05222 5308-82, frank.hasselfeld@syngenta.com
Havis	Neil	Scottish Agricultural College	Crop and Soil Research Group, SAC Edinburgh, West Mains Road Edinburgh, Scotland, EH9 3JG, U.K., +441315354136, Neil.Havis@sac.ac.uk

Last name	First name	Organ./Comp.	Address/ Phone/ E-mail
Heiser	Ingrid	Lehrstuhl für Phytopathologie, TU München, Wissenschaftszentrum Weihenstephan	Am Hochanger 2, 85350 Freising, Germany, +49 8161-714185, heiser@Irz.tum.de
Hemker	Reinhard	Limagrain-Nickerson GmbH	Salder Str. 4, 31226 Rosenthal, Germany, 05171-587914, Reinhard.Hemker@limagrain-nickerson.de
Hermann	Dietrich	Syngenta Crop Protection AG	Research Biology, WST 540.1.71, CH4332 Stein, Switzerland, +41-61-3231228, dietrich.hermann@syngenta.com
Herz	Markus	Bayerische Landesanstalt f. Landwirtschaft, Inst. f. Pflanzenbau u. Pflanzenzüchtung IPZ 2b	85354 Freising, Am Gereuth 6, Germany, 08161 71 3629, Markus.Herz@Lfl.bayern.de
Heß	Michael	Lehrstuhl für Phytopathologie, TU München, Wissenschaftszentrum Weihenstephan	Am Hochanger 2a, 85350 Freising, Germany, +49(8161)71-5041, m.hess@Irz.tum.de
Huss	Herbert	Agricultural Research & Education Centre Raumberg-Gumpenstein, Institute for Organic Farming	Field trial station Lambach-Stadl-Paura, Gmundnerstr. 9, A-4651 Stadl-Paura, Austria, +43 7245 20503 22, Herbert.huss@raumberg-gumpenstein.at
Jaiser	Heidi	Pajberg A/S	Grindsnabevej 25, Dyngby, DK-8300-Odder, Denmark, +45 87817644, Heidi.Jaiser@pajbjergfonden.dk
JØrgensen	Lise Nistrup	Danish Institute of Agricultural Sciences	Research Centre Flakkebjerg, 4200 Slagelse, Denmark, +45 89993652, Lisen.jorgensen@agrsci.dk
Kakau	Joachim	Landwirtschaftskammer Niedersachsen, Pflanzenschutzamt	Sedanstr. 4, 26121 Oldenburg, Germany, +49 441-801730, j.kakau@Iwk-we.de
Kempe	Harald	Saatzucht J. Breun GdbR	Saatzucht J. Breun GdbR, Amselweg 1, 91448 Herzogenaurach, Germany, +49 9132 78883, kempe@breun.de
Kirch	Gerd	Syngenta Crop Protection, Product Portfolio Management EAME, Technical Manager Fungicides	Schwarzwaldallee 215, Postfach, CH-4002 Basel, Switzerland, +41 61 323 79 52, gerd.kirch@syngenta.com

Last name	First name	Organ./Comp.	Address/ Phone/ E-mail
Koopmann	Birger	Plant Pathology and Protection Division, Georg-August-University, Göttingen	Grisebachstrasse 6 37077 Göttingen, Germany, +49-551-393776, bkoopma@gwdg.de
Kreye	Holger	Federal Biological Research Centre for Agriculture and Forestry	38104 Braunschweig Messeweg 11/12, Germany, +495312994545, h.kreye@bba.de
Krieg	Ulrich	Bayer CropScience Deutschland GmbH	Elisabeth-Selbert-Str. 4a, 40764 Langenfeld, Germany, +49 2173-2076-279 ulrich.krieg@bayercropscience.com
Liebermann	Bernd	Friedrich-Schiller-Universität Jena	Neugasse 23, 07743 Jena, Germany, 03641 949870, Bernd.Liebermann@uni-jena.de
Lüders	Wolfgang	Limagrain-Nickerson GmbH	Griewenkamp 2, 31234 Edemissen, Germany, +49 5176-989137, Wolfgang.Lueders@limagrain-nickerson.de
Makepeace	Joanne	John Innes Centre	John Innes Centre, Norwich Research Park Colney, Norwich NR4 7UH, U.K., 01603 450618, Joanne.makepeace@bbsrc.ac.uk
Maumene	Claude	Arvalis Institut du Vegetal	Station Experimentale, 91720 Boigneville, France, +33164992265, c.maumene@arvalisinstitutduvegetal.fr
Mausbach	Heinrich	Cebeco Saaten GmbH	Auestr. 5, 29352 Adelheidsdorf, Germany, + 49 5085-9810-0, cebecotechniknord@t-online.de
Miethbauer	Sebastian	Friedrich-Schiller-Universität Jena	Neugasse 23, 07743 Jena, Germany, Miethbauer.sebastian@uni-jena.de
Nesvadba	Zdenek	Agricultural Research Institute, Kromeriz, Ltd.	Havlickova 2787, Kromeriz, 767 01, Czech Republic, +420 573 317 166, nesvadba@vukrom.cz
Oxley	Simon	Scottish Agricultural College, Crop and Soil Research Group	SAC Edinburgh, West Mains Road Edinburgh, Scotland, EH9 3JG, U.K., +441315354094, Simon.Oxley@sac.ac.uk
Pinnschmidt	Hans	Danish Institute of Agricultural Sciences, Dept. of Crop Protection, Research Center Flakkebjerg	DK-4200 Slagelse, Denmark, +45-89993718, Hans.Pinnschmidt@agrsci.dk
Prigge	Günter	BASF Aktiengesellschaft	67117 Limburgerhof, Germany, +49 621-6027461, guenter.prigge@basf.com
Reinbrecht	Carsten	Pflanzenzucht SaKa GbR	Ranzin, Mecklenburg-Vorpommern, D-17495, Germany, 0049-38355-61593, Cr.pflanzenzuchtsaka@tele2.de

Last name	First name	Organ./Comp.	Address/ Phone/ E-mail
Reitan	Lars	Graminor AS	Volhaugvegen 97, N-7650 Verdal, Norway, +47 480 41 300, Lars.reitan@graminor.no
Rodemann	Bernd	Federal Biological Research Centre for Agriculture and Forestry	38104 Braunschweig, Messeweg 11/12, Germany, +49-531-2992550, B.rodemann@bba.de
Sachs	Edelgard	Biologische Bundesanstalt für Land- und Forstwissenschaft	Tremsdorfer Weg 9, 14558 Nuthetal, OT Bergholz-Rehbrücke, Germany, +49 33200-83315, egsachs@t-online.de
Salamati	Saideh	Midt-Norsk Plantevern AS	Kvithamar Research Centre, N-7500 StjØrdal, Norway, mnorsk@plantevern.no
Sattler	Ulf	Syngenta Agro GmbH	Am Technologiepark, 1-5, 63477 Maintal, Germany, +49 6181-9081346, Ulf.sattler@syngenta.com
Schützendübel	Andres	Plant Pathology and Protection Division, Georg-August-University, Göttingen	Grisebachstrasse 6, 37077 Göttingen, Germany, +49-551-393710, Aschuet1@gwdg.de
Semar	Martin	BASF Aktiengesellschaft	67117 Limburghof, Germany, +49-621 6027662, Martin.semar@central-europe.basf.org
Sindberg	Susanne	Department of Variety Testing Danish Institute of Agriculture Sciences	Teglverksvej 10, Tystofte, 4230 Skfelskor, Denmark, +45 58160600, susanne.sindberg@agrsci.dk
Spanakakis	Andreas	FR. Strube Saatzucht KG	Postfach 13 53 38358 Schöningen, Germany, +49 5354-809 0, spanakakis@fr-strube.de
Stabentheiner	Edith	Institute of Plant Sciences, Dept. of Plant Physiology, University of Graz	Schubertstrasse 51, 8010 Graz, Austria, +43-316-380-5637, Edith.stabentheiner@uni-graz.at
Stadler	Martin	Plant Pathology and Protection Division, Georg-August-University, Göttingen	Grisebachstrasse 6, 37077 Göttingen, Germany, Mortl82@yahoo.de
Stemberková	Lenka	SELGEN Co.	Stupice, 25084 Sibřina, Czech Republic, +420281012451, stemberkova@selgen.cz
Thomas	William	Scottish Crop Research Institute	Invergowrie, Dundee, DD2 5DA, UK, +44(0) 1382 568535, Bill.Thomas@scri.ac.uk
Tischner	Helmut	Bavarian State Research Center for Agriculture	Lange Point 10, 85354 Freising, Germany, +49/(0)8161/71-5652, Helmut.Tischner@LfL.bayern.de

Last name	First name	Organ./Comp.	Address/ Phone/ E-mail
v. Tiedemann	Andreas	Plant Pathology and Protection Division, Georg-August-University, Göttingen	Grisebachstrasse 6, 37077 Göttingen, Germany, +49-551-393701, atiedem@gwdg.de
Welz	Günter	FR. Strube Saatzucht KG	Postfach 13 53, 38358 Schöningen, Germany, +49 (0)5354-809 45, welz@fr-strube.de
Weyen	Jens	Saaten-Union Resistenzlabor GmbH	Hovedisser Str. 92, 33818 Leopoldshöhe, NRW, Germany, +49(0)5208 95 04 92, Weyen@saaten-union-labor.de