

Aus dem Institut für Phytopathologie
der Christian-Albrechts-Universität zu Kiel

**Rapid detection and quantification of *Cercospora beticola* in soil using PCR
and ELISA assays**

Dissertation
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Abbreviations

AFLP	Amplified fragment length polymorphism
bp	Base pair
c-DIV	The cumulative DIV
CLS	Cercospora leaf spot
DIV	Daily infection values
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	2'-deoxynucleoside 5'-triphosphate
ELISA	Enzyme linked immunoabsorbent assay
FCS	Fetal calf serum
HAT Medium	Hypoxanthine-aminopterin-thymidine medium
Ig	Immunoglobulin
IPM	Integrated pest management
ITS	Internal transcribe spacer
mAb	Monoclonal antibody
OD values	Optical density
pAb	Polyclonal antibody
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PDB	Potato dextrose broth
PEG	Polyethylenglycol
rH	Relative humidity
TE	Tris-EDTA
<i>T</i> _m	Melting temperature
TMB	Tetramethylbenzidine

Measures

μ	Micro
n	Nano
p	Pico

Units

C	Celsius
g	Gram
h	Hour (s)
min	Minute
s	Second
M	Mol
°	Degree

1 Introduction

1.1 Biology and epidemics of *Cercospora* leaf spot in sugar beet

Sugar beet (*Beta vulgaris* L.) was harvested for a long time as a vegetable and for medicinal use before its cultivation as a source of sugar. It was first cultivated for sugar production in Europe in the eighteenth century, however, it was probably known as early as 500 B.C. (Van Cleef 1915). By the turn of the century, the proportion of the world's beet crop used for sugar production rose from 14 % in 1853 to 65 % by 1900 (Poggi 1930).

Sugar beets are grown in more than 40 countries in Europe, Asia, the Middle East, northern Africa, and South and North America (Whitney and Duffus 1986). In Germany, sugar beet is grown on 362,000 ha and the average German sugar yield is 3.7 million tons (according to the data of "Statistik und Berichte, des Bundesministeriums für Ernährung, Landwirtschaft und Verbraucherschutz" in 2010/2011).

Sugar beet is considered to be an important source of crystalline sucrose, which is added to a wide range of foods, beverages and pharmaceuticals for its excellent properties (Cooke and Scott 1993). Additionally, sucrose is used in the manufacture of a range of products (e.g. polyurethane foams, high intensity sweeteners, vitamins and antibiotics) (Cooke and Scott, 1993). Sugar beet is also considered to be a source for molasses (Ulber *et al.* 2000) which are used as a component of animal feed (Scipioni and Martelli 2001). Molasses are also a carbon source for yeast production (Atiyeh and Duvnjak 2002), and are used in chemical and pharmaceutical production (Faurie and Fries 1999). Additionally, sugar beets are also grown for the seed production for the following year (Pospisil *et al.* 2000).

Cercospora beticola (Sacc.) causes *Cercospora* leaf spot (CLS) disease of sugar beet (*Beta vulgaris*) and most cultivated and wild species of *Beta*, *Spinacia oleracea* (spinach), and species of *Amaranthus*, *Atriplex*, *Chenopodium* and *Plantago* (Vestal 1933; Frandsen 1955; El-Kazzaz 1977 and Soyly *et al.* 2003). CLS is considered to be the most common and destructive foliar disease in sugar beet growing areas worldwide (Georgopoulos and Dovas 1973; Smith and Ruppel 1974), especially in regions with a humid, temperate climate (Cooke and Scott 1993). It can lead to reductions in sugar yield of up to 42% and 32% in root weight

(Shane and Teng 1992). In addition to reducing yield and quality of sugar beet, high frequent fungicide applications for CLS control add costs to producers. Additionally, the repeated uses of fungicide with high frequent applications causes fungicide-tolerant strains of *C. beticola* (Weiland and Koch 2004). *C. beticola* is the primary leaf pathogen of sugar beets in Germany, especially in regions with frequent rainfall and average daily temperatures of 20 to 25°C (Bleiholder and Weltzien 1972). The yield losses can range from 10 to 30% and the recoverable sugar yield reductions can reach up to 50%, economic losses may reach US\$1,500/ha (Shane and Teng 1985; Wolf *et al.* 1995).

C. beticola (Sacc.) was first described as a species of the genus *Cercospora* by Saccardo (1876). It is likely originated in central Europe and the Mediterranean area (Groenewald *et al.* 2005). Using the ITS sequences of a variety of *Cercospora* species contribute to make phylogenetic analyses which define *Cercospora* as a monophyletic section within the teleomorph genus *Mycosphaerella* (Stewart *et al.* 1999; Crous *et al.*, 2000, 2001, 2004; Goodwin *et al.* 2001; Pretorius *et al.* 2003).

The sexual state or teleomorph stage is unknown to occur in nature or laboratory. However, the mating type genes resulting from the PCR amplification of *C. beticola* genomic DNA have been observed and they are similar to those previously described in other species belonging to Mycosphaerellaceae (Groenewald *et al.* 2006). As the mating types in fungal populations which regularly reproduce sexually are similarly distributed (Milgroom 1996), there is a possibility that sexual reproduction happens in this asexually reproducing fungal species.

The data obtained from mating type analyses of *C. beticola* showed the presence of full length mating type genes in this fungus, indicating that these genes are still functional in *C. beticola*, but the teleomorph still needs to be discovered (Groenewald *et al.* 2006).

The symptoms of disease appear first on older leaves as individual spots followed by progressive appearance on younger leaves (Jacobsen and Franc 2009). Cotyledons also may become infected early in the season under conditions of high humidity and warm temperature. Leaf spots are small, ranging from 3-5 mm in diameter (Whitney and Duffus 1986) and are nearly circular (Ruppel 1986). Lesions are tan to light brown dark with brown or reddish-

purple margins. Individual spots on the leaves coalesce as the disease progresses (Fig. 1 A), and a mature lesion is characterized by completely necrotic tissue having fungal hyphae growing throughout (Cunningham 1928; Steinkamp *et al.* 1979). Also black dots, pseudostromata, are often visible in the centre of mature lesions (Fig 1 B). Under humid conditions, conidiophores are formed on the pseudostromata, and the leaf spots become grey along the production of conidia. Lesions also may form on petioles and appear elongated rather than circular (Giannopolitis 1978) and, moreover, circular lesions also have been reported on the portion of the root crown not covered by soil.

The primary inoculum sources within the disease cycle of the fungus are pseudostromata which may survive for 1-2 years (Pool and McKay 1916; McKay and Pool 1918; Canova 1959). However, conidia of *C. beticola* persist on infected leaf debris for only 1-4 months (Pool and McKay 1916). Infested seeds are also considered to be sources of inoculum (McKay and Pool 1918; Schürnbrand 1952), in addition to weed or wild beet hosts (Vestal 1933). Spread of conidia occurs mainly by rain-splash (Pool and McKay 1916; Carlson 1967). Some horizontal spread can also occur by wind (McKay and Pool 1918), irrigation water, insects and mites (McKay and Pool 1918; Meredith 1967). Figure 2 shows the disease cycle of *C. beticola*.

In Germany, some studies have reported high levels of variation during the onset and progression of *Cercospora* leaf spot on sugar beet (Wolf and Verreet 2002, 2005). Other studies reported that *C. beticola* has become resistant or has developed an increased tolerance to fungicides (Karaoglanidis *et al.* 2000; Weiland and Koch 2004). Groenewald *et al.* (2008) analysed the population structure and genotype diversity of 250 *C. beticola* isolates collected from western Europe, Iran and New Zealand using amplified fragment length polymorphism (AFLP), and demonstrated high levels of genetic variation among the *C. beticola* isolates tested.



Fig. 1: Disease progress of *Cercospora* leaf spot on sugar beet leaves (A). A single leaf spot caused by *C. beticola* on sugar beet with black dots consisting of conidia and conidiophores (B).

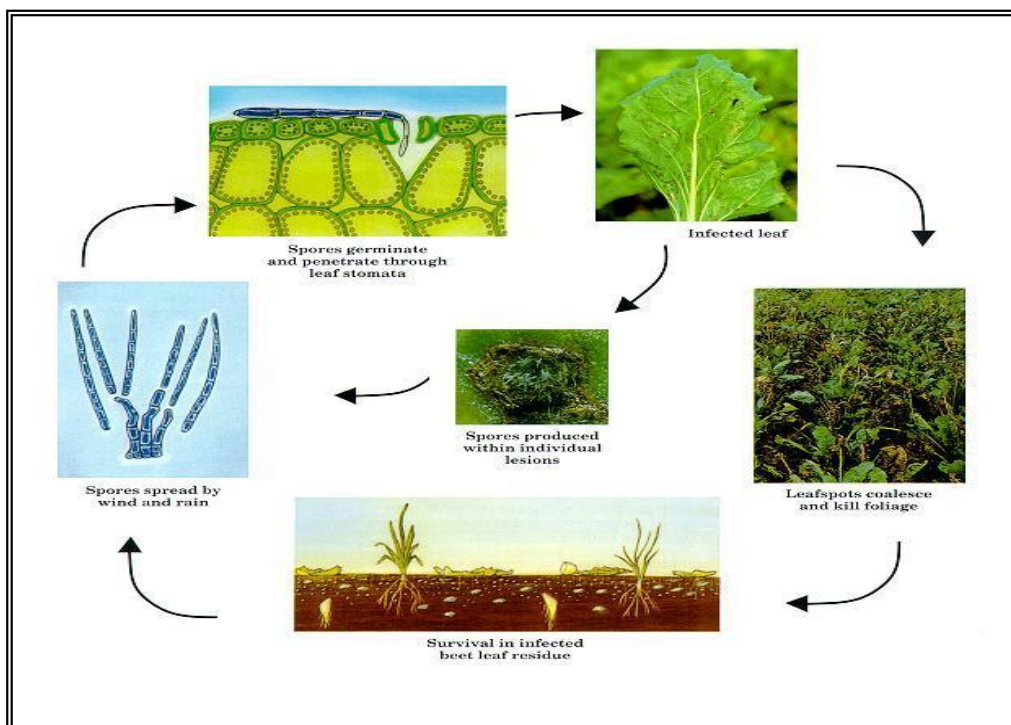


Fig. 2: Disease cycle of *Cercospora* leaf spot caused by *Cercospora beticola* (Jones and Windels 1991).

1.2 Integrated pest management (IPM) for *Cercospora* leaf spot (CLS) in sugar beets and its quaternary concept

In case of CLS becoming epidemic, applications of fungicides are the only mean to protect sugar beets from yield losses. Hoffmann *et al.* (1994) reported that under favouring conditions fungal pathogens may progress even from small inoculum potential to severe epidemics. Therefore, even though all other indirect control measures (crop rotation, soil cultivation, resistant cultivars, etc.) are optimized, there is only little chance to replace the use of fungicides if the cultures are aimed to earn optimal yield (Hoffmann *et al.* 1994). The integrated use of fungicides for reducing the chemical load on the environment is an important goal of IPM programs (Anonymous 2002), which has become a mainstream strategy for managing plant diseases over the last few decades (Jarvis 1992; Shea *et al.* 2000). Thus, the scheduled application of fungicides which is oriented to an average risk of disease should be substituted through a flexible managing of plant protection measures. As valid for many other fungal parasites, the damage values caused by *C. beticola* are variable depending on year, site and cropping practices (Wolf and Verreet 2003).

In Germany, Wolf and Verreet developed an effective IPM system for CLS which is flexible and primarily based on the epidemiology (Wolf *et al.* 2000; Wolf 2002; Wolf and Verreet 2002). They found that a single tool is not able to fulfill the demands of IPM, the reduction of fungicide applications to a necessary minimum on one hand and the optimization of yield factors on the other. Therefore, this model combines several IPM tools (Fig. 3), namely the prediction of epidemic onset (Wolf 2002; Wolf *et al.* 2004; Wolf and Verreet 2005a,b), fungicide action thresholds (Verreet *et al.* 1996; Wolf *et al.* 1998b, 2000, 2001 and 2004) and economic damage threshold (Wolf *et al.* 1998a; Wolf 2002; Wolf *et al.* 2004; Wolf and Verreet 2002) which is used for predicting yield losses (Wolf *et al.* 1998a, 2004; Wolf and Verreet 2002, 2003). In order to predict the disease onset, Wolf and Verreet introduced DIV values (daily infection values) derived from a 6-years study (1993-1998, 11 sites) in southern Germany, to describe the relationship between meteorological factors and onset of *Cercospora* leaf spot.

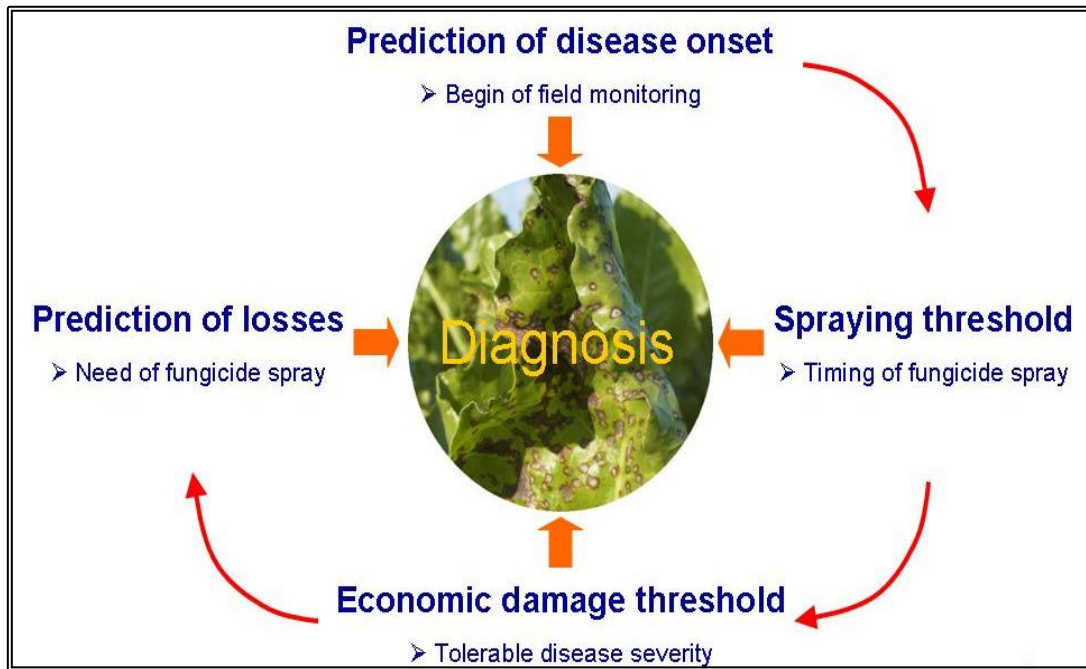


Fig. 3: Quaternary IPM concept with four IPM tools and the order to use them during a sugar beet growing season (Wolf and Verreet 2003; Wolf and Verreet 2005a; <http://www.ips-zuckerruebe.de/>).

Prediction of disease onset is the first tool of the IPM model, which provides information about the beginning of field monitoring. The influence of weather on the epidemic onset was assessed through the calculation of daily infection values (DIV) (Wolf and Verreet 2000; Wolf 2002). The authors found that c-DIV's derived from different years and sites could not explain the variation of epidemic onsets satisfactorily and therefore could not be used for direct indications of fungicide applications. On the other hand, the c-DIV may be used to calculate a risk of disease incidence. This is done by a negative prognosis, where the disease free period is determined by the minimum values of the cumulative DIV (c-DIV) when the disease onset occurred. Therefore, if this threshold is exceeded, the onset of disease cannot be excluded anymore. A field monitoring has to follow in order to determine the initiation of first symptoms.

The prediction of disease onset considers some factors which affect the disease incidence; meteorological factors, cropping measures (the cultivar susceptibility, crop rotation, soil cultivation). However, it doesn't consider the factor "inoculum in the soil" due to lack of detection methods. Additionally, the prediction is not precise enough to forecast the time of epidemic onset, thus it is defined as a "negative prognosis" and only the disease free period can be predicted. Therefore, effective methods of qualitative and quantitative detection of *C.*

beticola in soil could fill this gap of the IPM-model, where this study is focused on. So, using these methods, a better and more precise forecast of disease onset could be introduced if the amount of inoculum is included in the prediction.

The crucial point during the field monitoring is the diagnosis and quantification of disease in view of the incidence will exceed the fungicide action threshold. In order to optimize the efficiency of fungicide applications and define its exact time, successive stages of the epidemic were assessed for thresholds. An initial treatment should be carried out at disease severities in the range of 0.01 up to 0.2 %. At later epidemic stages, the application efficacy is decreasing to less than 80 %.

The economic damage threshold defines the tolerable disease level at the end of the season (Wolf *et al.* 1998a,b; Wolf and Verreet 2002). The relationship between disease severity and sugar loss, which shows a linear character, indicates a tolerance limit of 5% infected leaf area. Thus, after predictions of disease progress exceeding this level, benefits of fungicide applications are highly probable.

The prediction of sugar losses is necessary just when the spraying threshold is exceeded. For using this tool, other measurements must be considered such as the date when the economic threshold is exceeded, cultivar resistance and scheduled date of harvest. If the predicted disease severity exceeds the damage threshold before the scheduled harvest time, yield losses equal or greater than costs of applications are probable. Therefore, based on the relationship of disease initiation and disease severity at harvest time, risk periods were introduced. If action thresholds are exceeded during risk periods, fungicide treatments are necessary.

1.3 Disease diagnosis methods and the role of molecular techniques in plant pathogen management

Disease diagnosis is an important tool for the optimal use of fungicides and to practice IPM (Leonard and Fry 1986). In the past decades, conventional methods of plant pathogen detection were the only way for diagnostics, which relied to a large extent on morphological identification and symptom diagnosis. Although these methods are fundamental to diagnostics, their accuracy and reliability largely depend on skilled taxonomical expertise. Additionally, isolation and culturing of the organism *in vitro*, and sometimes pathogenicity

tests, are considered to be the basics of these methods (Singleton *et al.*, 1992), which need a lot of time and much efforts and depend on the ability of the organism to be cultured *in vitro*. Even when several hundred clones are examined, smaller populations (around or below 1%) may not be detected (Amann *et al.* 1995; Rapp and Giovannoni 2003). In addition, quantification based on these culturing techniques is considered relatively inaccurate and unreliable (Tsao and Guy 1977; Jeffers and Martin 1986; Thorn *et al.* 1996; Termorshuizen *et al.* 1998; Goud and Termorshuizen 2003).

For qualitative and quantitative assessment of soil microorganism, several indirect methods such as soil-dilution and plate counts are being used. However, these methods are labour intensive and require considerable taxonomic expertise. Additionally, these methods favour viable propagules and abundantly sporulating fungi as well as fast growing non-sporulating fungi (Curl and Truelove, 1985). As is well known, there exists no semi-selective medium for *C.beticola*.

In contrast, the molecular diagnostic techniques are increasingly being used for the early and precise detection and quantification of plant pathogens (Miller and Martin 1988). These include immunological (or serological) and nucleic acid-based techniques. Compared to conventional assays, these techniques are more specific, more sensitive, faster and more reliable than culture based detection methods (Weller-Alm *et al.* 2000 and Bonants *et al.* 2003) and can be performed by personnel with no taxonomical expertise. Additionally, these techniques are suitable for the detection of culturable as well as non-culturable microorganisms because they do not require culturing step.

Currently, serological techniques and polymerase chain reaction (PCR)-based assays, are considered to be the most widely used methods for the qualitative or quantitative detection of various pathogens including viruses, bacteria and fungi (Clark 1981; Henson and French 1993).

1.3.1 Serological techniques

Since almost 30 years, serological detection methods are being used for pathogen detection. Detection of viruses, which cannot be cultured *in vitro*, was the main objective of these methods. Serological techniques are based on the binding between diagnostic antibodies and specific antigenic determinants of the target pathogen. Enzyme-linked immunosorbent assay

(ELISA; Clark and Adams 1977) is considered to be the most commonly used serological technique; however, several other serological plant pathogen detection methods have been described (Lopez *et al.* 2003; Ward *et al.* 2004).

Compared with the other serological detection methods, ELISA has many advantages: its high-throughput capacity, promptness, low cost and the possibility to quantify the amount of target pathogen (Hampton *et al.* 1990; Schaad *et al.* 2001). Thus, ELISA has been widely applied for the identification of fungal pathogens and non-pathogens in host tissues (Aldwell *et al.* 1983; Unger and Wolf 1988).

Direct detection in soil is difficult due to the problems encountered in extracting fungal antigens from soil and to interference from non-specific soil contaminants. Retention of the antigen may occur on components of the soil solid phase by a range of physicochemical processes including electrostatic bonding by ion exchange after protonation and by hydrogen bonding, in particular between N-H groups and organic compounds (Dewey *et al.*, 1996). Due to the previous difficulties, there are a few studies of using ELISA assay for detection of fungal pathogens and non-pathogens in soil such as *Phytophthora* (Klopmeier *et al.* 1988), *Glomus* (Wright and Morton 1989), *Thanatephorus* (Dusunceli and Fox 1992) and *Rhizoctonia* (Thornton *et al.* 1993). Lartey *et al.* (2007) used ELISA to make a survey for detection of *C. beticola* in field soils. Furthermore, Caesar *et al.* (2007) applied ELISA for an accurate detection and quantification of *C. beticola* in field soil using polyclonal antibodies (pAb).

Polyclonal antibodies (pAb), which recognize multiple epitopes of the pathogen, have been used successfully for detecting many viruses; however, they do not always have a high degree of target specificity. Monoclonal or recombinant antibodies are considered to be the most accurate application of serological detection methods, which have a high degree of target specificity. Both techniques allow the selection of specific target epitopes to avoid “false positives”.

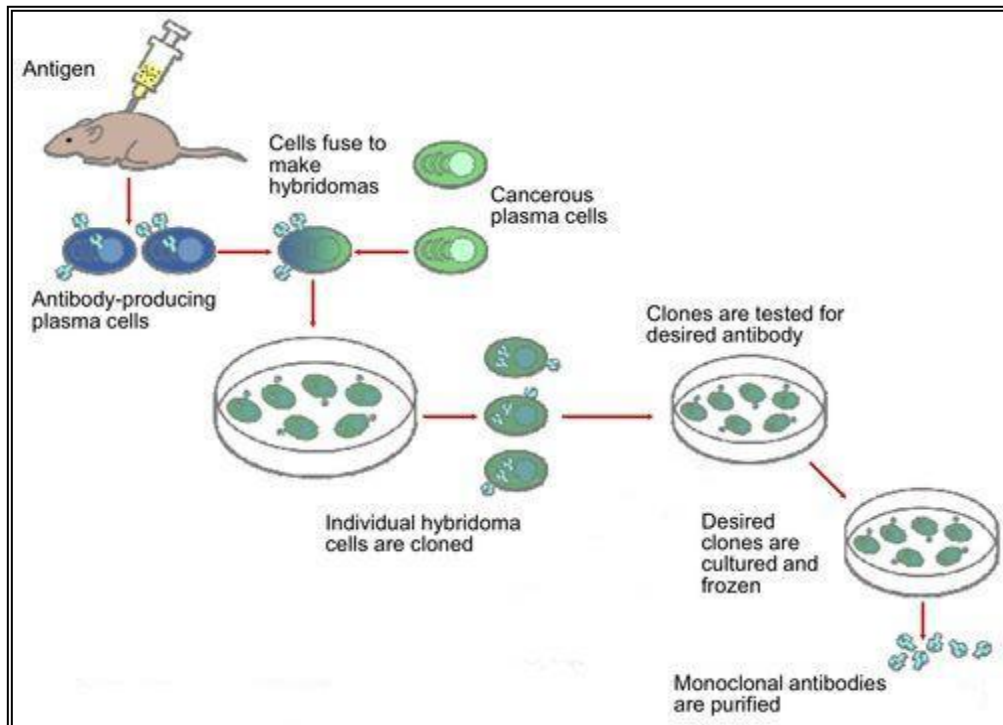


Fig. 4: Overview of monoclonal antibody production, described by Köhler and Milstein (1975).

The cell wall of fungi contains many different protein and carbohydrate components, which can elicit an immune response and which are considered to be a target for monoclonal antibodies (Werres and Steffens 1994). Antigen preparation has a great variability, although, three main antigen types are used for monoclonal antibodies production: (i) Mycelium homogenization (Dewey *et al.* 1989; Nameth *et al.* 1990; Fuhrmann *et al.* 1992; Burge *et al.* 1994; Bermingham *et al.* 1995; Hitchcock *et al.* 1997; Mitchell *et al.* 1997), where the mycelium is ground to a fine powder in liquid nitrogen using a mortar and pestle. The powder is solubilised and typically mixed with adjuvants for immunization. The monoclonal antibodies produced by this method can react with mycelium but not with spores of the same species (Werres and Steffens 1994). (ii) Intact spores or spore fragments (Xia *et al.* 1992; Hughes *et al.* 1999; Koistinen *et al.* 2000; Schmechel *et al.* 2003), where the spores are most commonly extracted directly from agar-plates by buffer-wash (Salinas and Schots 1994; Schmechel *et al.* 2006), and then emulsified with adjuvant for immunization. (iii) Washing the surface of spores or mycelium with buffer (Dewey and Meyer 2004; Thornton *et al.* 2002), where surface components are often proteins, toxins and polysaccharides, which are either secreted by the fungi or associated with the pathogen surface (Werres and Steffens 1994).

1.3.2 PCR-based methods

PCR is one of the most efficient and useful techniques employed for nucleic acid detection (Legay *et al.* 2000) and a powerful tool in molecular biology to exponentially amplify many copies of a specific segment of DNA (Campbell 1996). DNA sequences are amplified over 20-30 reaction cycles by repeating the replication cycle, which increases the number of target sequences exponentially in each cycle (Campbell 1996; Henson and French 1993, Fig. 5).

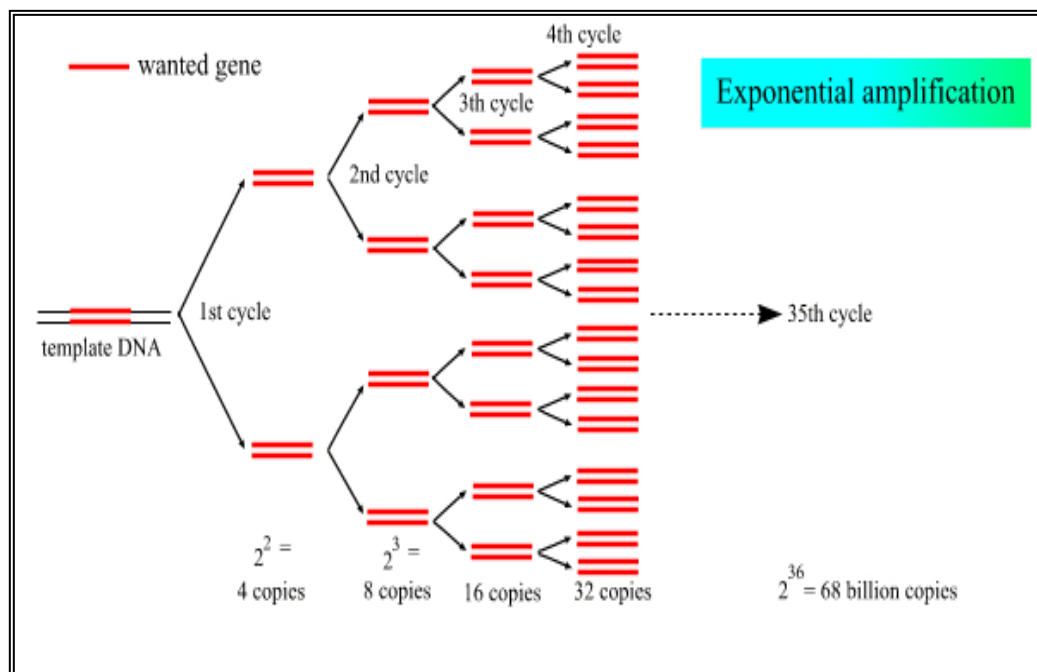


Fig. 5: The exponential amplification of DNA by PCR (Vierstraete 1999).

The method includes three essential steps: (i) DNA denaturation by melting of target DNA, (ii) annealing of forward and reverse primers to the denatured DNA strands, and (iii) primer extension by a thermostable DNA polymerase. Theoretically, this method enables the amplification of a single copy of a target DNA sequence to produce millions of copies of the target gene fragment (Bohm *et al.* 1999; Mullis and Faloona 1987, Fig. 6).

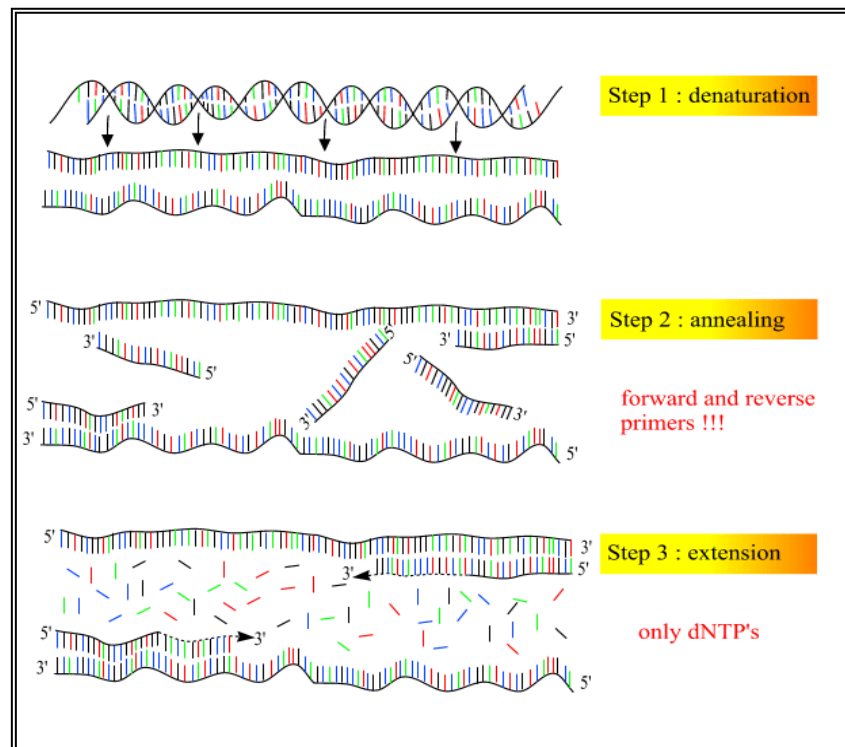


Fig. 6: The steps of PCR amplification (Vierstraete 1999).

Compared with traditional diagnostic methods, PCR has several advantages: (1) due to its high sensitivity, pathogens can be detected presymptomatically; (2) it doesn't need culturing steps before the detection of microorganisms, which makes it suitable for the detection of culturable as well as non-culturable microorganisms; (3) high sensitivity detection of a single target molecule without using radioactive probes; (4) it can be performed by personnel with no taxonomical expertise; and (5), it can be used for the simultaneous detection of several different pathogens using multiplex PCR.

The polymerase chain reaction (PCR) had not been used for the detection of plant pathogens until 1990, although it had been successfully applied for the identification of animal microorganisms (Rollo *et al.* 1990). In the last decade, this method has been widely applied for the detection of various viral, bacterial and fungal plant pathogens (Annamalai *et al.* 1995) and many reports describe specific applications of PCR technology in plant pathology (Haas *et al.* 1995; Zijlstra *et al.* 1997; Judelson and Tooley 2000; Amiri *et al.* 2002; Nie and Singh 2003). By now, at least 178 phytopathogenic fungal species of 97 genera have been successfully identified by PCR technique (from the database of PCR primers for phytopathogenic fungi, <http://www.sppadbase.com/>).

PCR primers can be designed for a unique DNA sequence, which allows to determine the presence or absence of that sequence, and thus of the specific organism. There are different methods which can detect the presence of amplified DNA, traditional detection by gel electrophoresis, colorimetric and fluorimetric assays (Mutasa *et al.* 1996; Fraaije *et al.* 1999). The high level of sensitivity of PCR-based detection methods give it the ability to detect minute quantities of pathogen DNA, even the amount derived from a single fungal spore (Lee and Taylor 1990).

Quantification of the amount of pathogen DNA, supplying the information required for disease management decisions, and for monitoring the effects of these decisions, has also been pursued using PCR-based methods. Theoretically, the exponential nature of PCR allows the amount of product to be used for the calculation of the initial amount of DNA at any time point in the reaction. However, when the plateau level is reached, since the reaction has stopped and no more products are being made, the amount of product is no longer proportional to the original amount of template. Competitive PCR overcomes this problem, where a target DNA can be quantified, which is based on the co-amplification of target DNA and competitor DNA, both with the same primer pair (Siebert and Larrick 1992) and known amounts of reporter or internal control sequences are included in PCR reactions (Diviacco *et al.* 1992; Hu *et al.* 1993). By comparing the relative amounts of target and competitor PCR product on agarose gel, the amount of target DNA can be determined subsequently. This method has been successfully used for the quantification of *Verticillium* wilt pathogens (Hu *et al.* 1993).

1.3.2.1 Real-time PCR

Conventional PCR is based on end-point detection of amplified products, where there is no possibility to monitor the reaction from start to end in order to quantify the amount of initial DNA, or to calculate the efficiency during the amplification process. In contrast, real-time PCR is considered to be a powerful technique for the quantification of a target DNA (Higuchi *et al.* 1992, 1993; Heid *et al.* 1996), which allows the monitoring of PCR products at each reaction cycle and quantifies the initial amount of the template DNA with high specificity and sensitivity without the need of further processing such as gel by electrophoresis and therefore avoids the use of hazardous dyes like ethidium bromide (Abd-Elsalam 2003; Schena *et al.* 2004).

The fluorescent reporter is considered to be the basics for the quantification using real-time PCR (Lee 1993; Livak *et al.* 1995), where DNA amplification can be monitored during each cycle based on the emission of fluorescence during the exponential phase (Fig. 7) (Heid *et al.* 1996; Mackay *et al.* 2002). Generally, the initial amount of target DNA is related to a threshold cycle (Ct), which is defined as the cycle number at which fluorescence emission exceeds the set threshold. Target DNA in unknown samples is quantified using a standard curve which relates threshold cycles (Ct) of dilution series of a known amount of target DNA to its amount of DNA. Thus, real-time PCR is considered to be more accurate since it quantifies the initial DNA based on the Ct value compared with the other quantitative PCR methods which quantify DNA at the endpoint (Qi and Yang 2002).

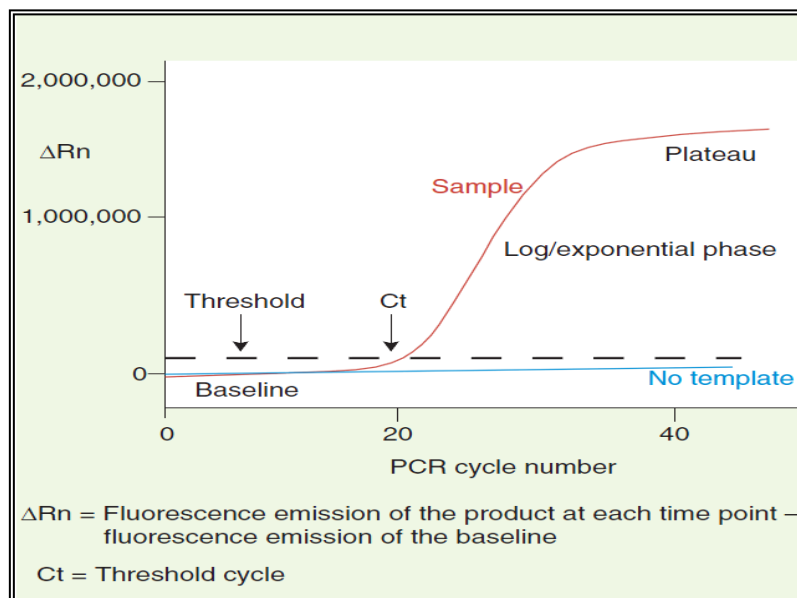


Fig. 7: Model of a single amplification plot illustrating the nomenclature commonly used in real-time quantitative PCR (Arya *et al.* 2005).

Amplicons can be detected using two main systems, which can be divided into either amplicon non-specific (Wittwer *et al.* 1997; Morrison *et al.* 1998) and amplicon specific methods (Holland *et al.* 1991; Livak *et al.* 1995; Wittwer *et al.* 1997; Livak 1999; Thelwell *et al.* 2000; Mhlana and Malmberg 2001; Schena *et al.* 2004) using DNA-binding dyes and sequence specific probes, respectively. SYBR green is one of the most frequently used real-time PCR dyes. Its uses are based on binding between double stranded DNA and DNA

binding dye. This dye exhibits little fluorescence in solution and becomes intercalated within the double stranded DNA product during primer extension and polymerization, resulting in an increase in detected fluorescence (Fig. 8).

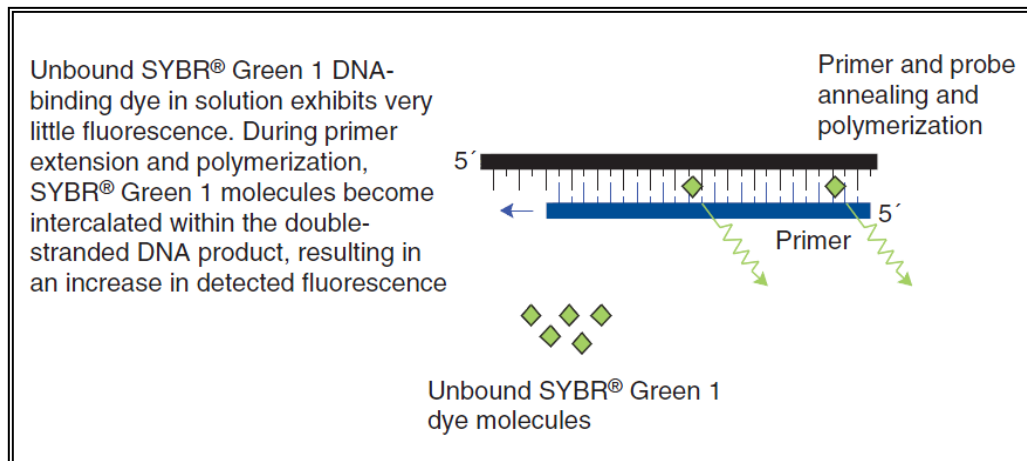


Fig. 8: Double stranded DNA-intercalating agents/DNA-binding dyes, e.g., SYBR[®] Green 1 (Arya *et al.* 2005).

SYBR Green is a more straightforward and less expensive approach compared to using sequence specific probes, but it is also less specific since the dye binds to all double stranded DNA present in the sample and cannot discriminate the target amplicon from other nonspecific amplicons and primer dimers (Ririe *et al.* 1997). Additionally, the formation of primer dimers may give rise to double stranded DNA fragments, which are detected by SYBR green. Thus, the specificity of the fluorescence signal relies on the design of the primers and the optimization of reaction conditions (Mackay *et al.* 2002; Papp *et al.* 2003).

In contrast, amplicon sequence specific methods, which include TaqMan probes (Livak *et al.* 1995), molecular beacons (Tyagi and Kramer 1996) and scorpions (Whitcombe *et al.* 1999), offer the advantages of increased specificity, certainly in combination with specific primers, and reducing signals due to mispriming or primer-dimer formation (Livak *et al.* 1995)

In recent years, the applications of real-time PCR assays have been widely used for accurate detection and/or quantification of specific plant pathogens (Bohm *et al.* 1999; Boonham *et al.*

2002; Winton *et al.* 2002; Mercado-Blanco *et al.* 2003). These applications include: (1) monitoring of disease development of pathogens in fields (Brouwer *et al.* 2003; Qi and Yang 2002; Gachon and Saindrenan 2004); (2) evaluation of the plant varieties resistance for the plant pathogens (Fraaije *et al.* 2001; Hietala *et al.* 2003; Vandemark *et al.* 2003); (3) evaluation of fungicide resistance and efficacy (Nascimento *et al.* 2003).

1.3.2.2 Application of PCR-based methods in *C. beticola* diagnosis

Groenewald *et al.* (2007) has applied the PCR assay to develop polymorphic microsatellite and single nucleotide polymorphism markers for *C. beticola*. In this study a total of 550 clones were sequenced, analyzed and screened for microsatellite repeats. Four clones that contain eight or more perfect repeats were found, and an additional five that contain nonperfect repeats were selected. Knowledge regarding the population structure of this important plant pathogen can be improved through uses of these nine markers which provide polymorphic genetic markers and can be applied to additional populations with larger sample sizes.

Analysis of fungicide resistance in the plant pathogen is being considered an effective tool for evaluating fungicide efficiency. Malandrakis *et al.* (2011) has used allele specific primers in conventional and real time PCR reactions for the qualitative and quantitative detection of the G143S QoI resistance mutation in *C. beticola*. The molecular diagnostic techniques developed in this study should aid QoI resistance management in *C. beticola* by providing the means for early detection of the G143S resistance mutation even at very low frequencies.

1.4 Objectives of this study

C. beticola is considered to be the most common and destructive foliar disease in sugar beet growing areas worldwide. Early inoculum detection methods are important tools for disease control by using fungicides. The IPM sugar beet model includes a prediction of the disease onset, but it does not consider the factor of inoculums detection due to the missing of detection methods. Thus, the overall objective of the research described in this thesis was to develop effective methods for the qualitative and quantitative detection of *C. beticola* in soil which should provide an effective tool for improving the IPM model. For achieving this objective, the following steps were carried out:

1. Development of specific monoclonal antibodies (mAbs) and of a simple and efficient ELISA protocol.
2. Development of specific primers.
3. Proving of developed mAbs and primers for their ability to detect *C. beticola* qualitatively and quantitatively in artificially infested soil, where the disease amount was known.
4. Inoculum degradation study under controlled conditions in climate chamber by quantification of *C. beticola* using adapted real-time PCR and ELISA-methods.
5. Appliance of new methods in the field: Detection and quantification of *C. beticola* in naturally infested field soil from different locations.
6. Study of inoculum degradation in naturally infested soil using real time PCR and ELISA.

2 Materials and methods

2.1 Pathogenicity test

Pathogenicity tests were carried out for comparison between different isolates of *C. beticola* depending on their Pathogenicity. These tests were carried out under standard conditions in climate chamber at 25 to 22° C (day/night, respectively), 14-16 h photoperiod (following the method of Shane and Teng 1983) and 50% rH at the Institute of Phytopathology, University of Kiel. Twenty-five cm diameter pots were filled with potting soil. Three replicate pots were used for each *C. beticola* isolate and one seed of the susceptible sugar beet cultivar Klarina (KWS), was planted in each pot. Sugar beet plants received all their water sufficient through the pre-inoculation periods. Two months old sugar beet plants were inoculated with spore suspensions (10,000 spores/ml) using an atomizer such that both sides of each leaf were lightly wetted (following the method of Shane and Teng 1983). Twenty isolates from different countries were tested including twelve isolates from Germany, four isolates from USA (the United States Department of Agriculture) and four isolates from Egypt (Agriculture Research Centre). Inoculated plants were then covered with polyethylene bags for 3 days. Disease severity (in %) was recorded 2 weeks after inoculation according to the disease rating scheme of Shane and Teng (1992).

Table 1: Spot-percentage scale for assessment of *Cercospora* leaf spot of sugar beet (Shane and Teng 1992)

Class	Average number of spots per leaf	Disease severity (%)
1	0	0.00
2	< 1 ^a	0.01
3	1-5	0.10
4	6-12	0.35
5	13-25	0.75
6	26-50	1.50
7	50-75 ^b	2.50

^a This category is used for plants where the disease was seen but the average number of spots per leaf is less than one.

^b For each plant, a minimum of five leaves were examined for *Cercospora* leaf spot. At low severities (<3%), each plant was categorized according to spots per leaf as given above; at high disease intensities, disease severity was estimated by comparison of leaves with standard area diagrams (Fig. 9).

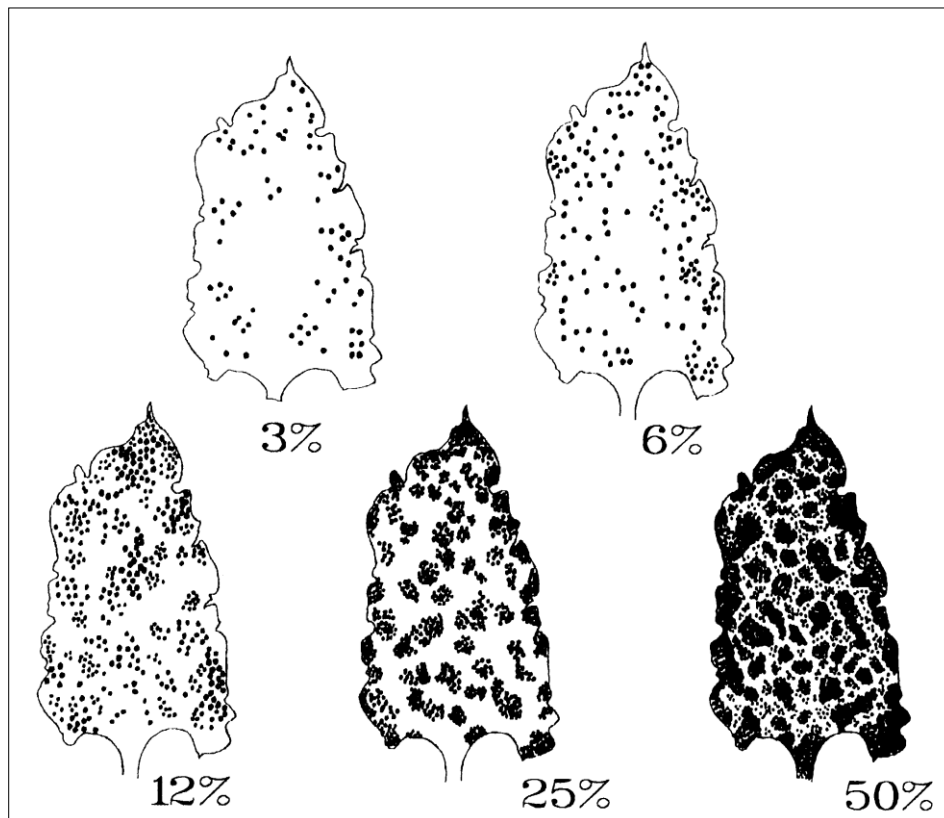


Fig. 9: Standard area diagrams for assessment of disease severity for *Cercospora* leaf spot of sugar beet (Shane and Teng 1992).

2.2 Preparation of the fungal species

The fungal species and isolates used in this study are listed in Tables 2 and 3. They were provided by the Institute of Phytopathology, University of Kiel with the exception of four isolates of *C. beticola* which were provided by Dr. Robert T. Lartey from the United States Department of Agriculture. Additionally, nine isolates of *C. beticola* were provided by Dr. Mohammad Yassin in the Institute of Phytopathology, Agriculture Research Centre, Giza, Egypt.

Twenty-five isolates of *C. beticola* and the other fungi pure cultures were grown on potato dextrose agar (PDA) plates (24 g potato dextrose broth and 15 g agar in 1000 ml autoclaved distilled H₂O). The plates were kept at 25° C until the mycelium covered most of the surface. Then, a disk of every isolate was transferred to potato dextrose broth (PDB) liquid medium (the same as PDA except agar). This inoculation induced the production of fresh mycelium within 7-9 days at 25° C. The mycelium of each isolate was then harvested from the liquid medium by filtration, and kept at -20 ° C until further use for DNA extraction.

Table 2: *Cercospora beticola* isolates used in this research study from sugar beets and their origin

No.	Isolate	Origin	No.	Isolate	Origin
1	Cb	Germany	14	C2	USA
2	Cb1	Germany	15	S1	USA
3	Cb2	Germany	16	S2	USA
4	Cb3	Germany	17	CbE1	Egypt
5	Cb4	Germany	18	CbE2	Egypt
6	Cb5	Germany	19	CbE3	Egypt
7	Cb6	Germany	20	CbE4	Egypt
8	Cb7	Germany	21	CbE5	Egypt
9	Cb9	Germany	22	CbE6	Egypt
10	Cb10	Germany	23	CbE7	Egypt
11	Cb12	Germany	24	CbE8	Egypt
12	Cb13	Germany	25	CbE9	Egypt
13	C1	USA			

Table 3: Fungal species used in this research

No.	Species	No.	Species
1	<i>Fusarium solani</i>	15	<i>Verticillium dahliae</i>
2	<i>F. moniliforme</i>	16	<i>Septoria petroselini</i>
3	<i>F. avenaceum</i> Rg 7/5	17	<i>Microdochium nivale</i>
4	<i>F. oxysporum</i> f.sp. <i>vasinfectum</i>	18	<i>Stagonospora nodorum</i>
5	<i>F. sporotrichioides</i>	19	<i>Gliocladium catenulanum</i>
6	<i>F. graminearum</i> 37	20	<i>Pseudocercospora anguioides</i>
7	<i>F. antheophilus</i>	21	<i>Rhizopus</i> spp.
8	<i>F. culmorum</i>	22	<i>Pyrenophora graminea</i> 15
9	<i>F. tricinctum</i>	23	<i>Botrytis</i> spp.
10	<i>Rhizoctonia solani</i> (AG2-1)	24	<i>Cladosporium</i> spp.
11	<i>Phytophthora ramorum</i>	25	<i>Alternaria alternata</i>
12	<i>Phytophthora infestans</i>	26	<i>Pseudocercospora herpotrichoides</i> var. <i>acuformis</i>
13	<i>Macrophomina phaseolina</i>	27	<i>Cyathus striatus</i>
14	<i>Chaetomium</i> spp.	28	<i>Cyathus olla</i>

2.3 DNA extraction

2.3.1 DNA extraction from fungal species and isolates

The method of Cenis (1992) with a slight modification was used to extract DNA from the mycelium. The mycelium was ground in liquid nitrogen and 50-100mg of the mycelium grinded was transferred to a 2 ml micro-tube. Five hundred μ l extraction buffer (200 mM Tris-HCl (pH 8.5), 250 mM NaCl, 25 mM EDTA, 0.5% (w/v) SDS) was then added with 10 μ l (10 μ g/ μ l) RNase A (Fermentas Molecular Biology, St. Leon-Rot, Germany) and mixed well. The micro-tubes were then incubated at 65° C in a water bath for 10 min, cooled down to room temperature, 170 μ l 3 M sodium acetate (pH 5.2) was added and the micro-tubes were incubated at -20° C for 5 min. The micro-tubes were then centrifuged at 15000 g for 5

min. The upper aqueous phase was decanted into a fresh centrifuge tube, an equal volume of isopropanol (100%) was added, then mixed well and the micro-tubes were stored at room temperature for 5 min. They were then centrifuged at 15000 g for 5 min at 4° C. The DNA pellet was washed with 500 µl 70 % ethanol, vacuum dried and dissolved in 50 µl TE buffer (100 mM Tris-HCl, 1 mM EDTA, pH 7.5). The DNA was then stored at -20° C for later PCR reaction.

2.3.2 DNA extraction from soil

DNA was extracted from soil samples using The Power Soil™ DNA Isolation Kit (MO BIO Laboratories, Inc. 2746 Loker Avenue West, Carlsbad, CA 92010, USA, obtained from Dianova, 20354 Hamburg, Germany) as described by manufacturer's instructions with a minor modification. About 0.25 g of each soil sample was added to manufacturer provided Power Bead Tubes, and gently vortexed. Sixty µl of the pre-warmed solution C1 was then added to each soil sample and mixed well. The mixture was incubated for 15 min at 70°C in a water bath. The Power Bead Tubes containing the samples were then vortexed at maximum speed for 10 min on tissue lyser (RETSCH, Qiagen TissueLyser), then centrifuged for 30 seconds at 10,000 g at room temperature. The supernatant (400 to 500µl) was then transferred into manufacturer-provided clean 2 ml collection tubes, and 250µl of solution C2 was added to each sample, mixed well by vortexing for 5 seconds and incubation for 5 min at 4°C. Then the sample was centrifuged for 1 min at 10,000 g at room temperature. About 600µl of the supernatant were then carefully transferred into clean 2 ml collection tubes, and 200µl of solution C3 was added to each sample, mixed well by vortexing for 5 seconds, incubation for 5 min at 4°C and centrifugation for 1 min at 10,000 g at room temperature. About 750µl of the supernatant were then carefully transferred into clean 2 ml collection tubes, 1200µl of solution C4 was added to each sample and mixed well by vortexing for 5 seconds. From this mixture, approximately 675µl were loaded onto a spin filter (provided by the manufacturer) and centrifuged for 1 min at 10,000 g at room temperature. The filtrate was discarded and an additional 675µl of the mixture was transferred to the spin filter and centrifuged for 1 min at 10,000 g at room temperature. The filtrate was discarded and the remaining supernatant was transferred onto the spin filter and centrifuged for another 1 min at 10,000 g at room temperature. Onto the spin filter, 500µl of solution C5 was added and centrifuged for 30 seconds at 10,000 g at room temperature. The filtrate was discarded and centrifuged again at

room temperature for an additional 1 minute at 10,000 *g*., The spin filters were then carefully transferred to clean 2 ml collection tubes. Finally, 100µl of solution C6 was added to the centre of the spin filters membrane, centrifuged for 30 seconds at 10,000 *g* at room temperature and stored at -20° until use.

2.3.3 Isolation of DNA fragments from agarose gel for sequencing

The QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) was used to extract DNA fragments from the agarose gel. The amplified DNA fragments were firstly electrophoresed on a horizontal agarose gel and visualized under UV light, and then using a clean, sharp scalpel, the DNA fragments were cut from the gel and placed into a microcentrifuge tube. The gel slice was weighed and three volumes of binding buffer QG were added to one volume of gel (100 mg ~ 100 µl) and incubated at 50°C for 10 min (or until the gel slice has completely dissolved). The tubes were mixed by inverting the tube every 2-3 min during the incubation to help the dissolving. After complete dissolving, one gel volume of isopropanol (100%) was added to the sample and mixed well. The sample was then transferred into the QIAquick column placed in a 2 ml collection tube to bind DNA, and centrifuged for 1 min at 11.000 *g* at room temperature. The flow-through was discarded, the QIAquick column was placed back in the same collection tube and 500 µl of buffer QG was added to QIAquick column to remove all traces of agarose. The column was centrifuged for 1 min and the flow-through was discarded. Then, 750 µl of washing buffer PE was added to QIAquick column and centrifuged for 1 min. The flow-through was discarded and centrifuged for an additional minute to ensure complete removal of the buffer. The QIAquick column was then placed into a clean and sterile 1.5-ml microcentrifuge tube. To elute the purified DNA, 50 µl of elution buffer (EB) was added and the column was centrifuged for 1 min at 11.000 *g*. The isolated DNA was air dried (S-Concentrator, Helmut Saur Laborbedarf, Reutlingen, Germany) until the tubes had completely dried. The samples were then sent to Eurofins MWG Operon (Ebersberg, Germany), to get the DNA sequence of the samples.

2.4 Design of specific primers

2.4.1 Search of *C. beticola*- specific sequences in NCBI-GenBank

Firstly, three different sequences of *C. beticola*; a 469-bp rDNA sequence (AY343371), a 967-bp cytochrome b (cytb) mRNA sequence (EF176921) and a 1195-bp actin gene (AF443281) were identified in the NCBI-GenBank (www.ncbi.nlm.nih.gov/). The selected sequences were then used for the design of specific primers using the internet program3 (<http://frodo.wi.mit.edu/primer3/input.htm>).

2.4.2 Specificity and sensitivity tests of selected primer

The specificity of design primer sets was tested by using the genomic DNA of *C. beticola* of twelve isolates from Germany, nine isolates from Egypt, four isolates from USA and 28 other fungal pathogens (Tables 2 and 3). The sensitivity of the specific primer sets was tested with different concentrations of *C. beticola* genomic DNA (100 ng, 10 ng, 1 ng, 100 pg, 50 pg, 10 pg, 5 pg, 1 pg, 0.5 pg, and 0.1 pg).

2.4.3 Conventional and real-time PCR conditions

2.4.3.1 Conventional PCR conditions

The PCR was carried out under the following conditions: initial denaturation at 95° C for 5 min followed by 35 cycles of 94° C for 30 s, 60° C for 30 s, 72° C for 45 s, with a final 10 min extension at 72° C. For the specificity test of the primer set Cb-actinF1/ Cb-actinR2, the PCR was carried out under the following conditions: initial denaturation at 95° C for 2 min followed by 28 cycles of 94° C for 1 min, 65° C for 30 s, 72° C for 1 min, with a final 5 min extension at 72° C. The PCR reaction contained 2 µl of template DNA (10ng/ µl), 0.2 µM from each primer (Eurofins MWG Operon, Ebersberg, Germany), 1.5 mM MgCl₂, 0.5 mM dNTPs and 0.5 µl of Draem Taq DNA polymerase (Fermentas Molecular Biology, St. Leon-Rot, Germany) in 5 µl of the manufacturer's reaction buffer.

2.4.3.2 Real-time PCR conditions

The real-time PCR (Applied Biosystems, 7300 Real Time PCR System) was carried out at 95° C for 5 min followed by 35 cycles of 94° C for 30 s, 60° C for 30 s, 72° C for 45 s, followed by dissociation run from 60 to 95° C. The real-time PCR reaction contained 0.6 µl (0.4 µM) of each primer, 7.5 µl SYBR Green mix (Qiagen Quantitect) and 2 µl of template DNA (10ng/ µl) with a total of 15 µl reaction volume. The quantified DNA of soil samples obtained by real-time PCR (in ng/µl) was then converted to ng/g soil according to the following formula: DNA (ng/g soil) = {DNA (ng/µl) derived from real-time PCR / 2 (each real-time PCR reaction containing 2 µl of sample DNA)} x total extracted DNA (100 µl) x 4 (soil weight of sample is 0.25 g).

2.5 Monoclonal antibody production and their specificity test

2.5.1 Cell wall preparation (antigen)

The production of monoclonal antibodies was carried out at the Institute of Biochemistry, University of Kiel. Mycelium of *C. beticola* (Cb12) was grind in liquid nitrogen, then homogenized three times in phosphate buffered saline (PBS, 8.00 g NaCl, 0.20 g KCl, 1.44 g Na₂HPO₄, 0.24 g KH₂PO₄ per l, pH 7.4) using a homogenizer (DIGITAL Sonifier® UNITS Model S-250D, Branson). Each cycle of homogenization was performed on ice twice at 60 sec intervals. The mixture was then centrifuged for 30 min at 20,000 g and 4° C. The sediment was discarding and the supernatant was centrifuged again for 1 h at 100,000 g and 4° C (Optimal TLX ultracentrifuge Beckman, rotor TLA 55). The supernatant was discarded, the membrane was resuspended in PBS, divided into aliquots and stored at -20° C until use.

2.5.2 Immunization

The monoclonal antibodies against cell wall of *C. beticola* were produced by the method of Köhler and Milstein (1975). Balb/c mice were initially immunized intraperitoneally (i.p.) with 80 µg of purified *Cercospora* cell wall in PBS emulsified with 40 % of Gerbu adjuvant MM (Gerbu, Wieblingen, Germany). On days 14 and 21, the mice were injected i.p. with 50 µg of purified *Cercospora* cell wall in PBS emulsified with 20% of the adjuvant. On the last three

days 28 , 29 and 30, the mice were injected with 50 µg *Cercospora* cell wall in PBS without adjuvant, while the fusion was done on day 31 (Table 4).

Table 4: Immunization scheme for production of monoclonal antibodies against the cell wall of *Cercospora beticola*

Day	0	14	21	28	29	30	31
Antigen [µg]	80	50	50	50	50	50	Fusion
Adjuvant [%]	40	20	20	0	0	0	

2.5.3 Collection of blood samples

The mouse was exposed to an infrared lamp for 10-15 min in a small container, to increase the blood flow to the tail. The tail was swabbed with antiseptic and several drops of blood were collected using a sterile scalpel on one of the lower veins that should be visible, then the mouse was returned back to its cage. The tubes of blood samples were then incubated for 1 h at 37 °C and shaken several times to dislodge the clot. The tubes were then transferred to 4 °C for 2 h or overnight and centrifuged at 10,000 g for 10 min at 4 °C, the pellet was discarded and the supernatant was transferred to a new tube and centrifuged again for 10 min. In a new tube, the supernatant was transferred and preserved by adding 0.02 % sodium azide before being frozen at -20 °C until it was tested by ELISA against *C. beticola*. Different dilutions were prepared from the blood samples. When the minimum dilution was good enough (1:100,000) the animal was euthanized to generate monoclonal antibodies.

2.5.4 Cell fusion

The mouse was euthanized and alcohol was used to swab the abdominal area. The spleen was removed from the abdomen using sterile forceps and scissors and placed into a sterile Petri dish containing 10 ml of serum free RPMI 1640 medium (Biochrom, Berlin). Using syringe needle and sterile forceps, the splenocytes were passed through a sterile Falcon 0.40 µm cell filter (Sarstedt, Nümbrecht, Germany), then transferred to a 15 ml tube and centrifuged for 5 min at 1300 g. After discarding the supernatant and washing the pellet of splenocytes twice in serum free medium, the pellet was resuspended in 10 ml medium and using a

haemocytometer, the viable lymphocytes were then counted. At the same time, Ag8.653 myeloma cells (Biochemistry Institute, University of Kiel) were washed twice with serum free medium. The myeloma cells were then centrifuged for 5 min at 1.100 g before being resuspended in 10 ml medium and counted. The myeloma cells and spleen were mixed at a ratio of 2:1-5:1 in a 50 ml tube and centrifuged for 5 min at 1.100 g. Using a Pasteur pipette, the supernatant fluid was removed as completely as possible, then the pellet was gently disrupted by tapping the bottom of the tube. Then, 1 ml of warmed 50 % PEG 1500 (w/v) (Roche Diagnostics, Germany) was added slowly to the pellet and mixed well by tapping the side of the tube. The mixture of PEG/cell was then gradually diluted with 5 ml serum free medium for 5 min, and after that with 25 ml serum free medium for 5 min under continuous and gentle swirling of the tube. Finally, the tube was centrifuged for 5 min at 1.100 g. After removing the supernatant, the fused cells were gently resuspended in 400 ml volume of standard RPMI 1640 medium supplemented with 10 % Fetal calf serum (FCS) (Invitrogen, Karlsruhe, Germany), HAT selection medium (Sigma, Taufkirchen, Germany), 2.5 ml Ciprobay (Biochemistry Institute, University of Kiel), 5 µg/ml amphotericin B (Merck Biosciences, Darmstadt) and 10 % J774 supernatant (Biochemistry Institute, University of Kiel). After distribution of the cell suspension in fusion plates (Greiner, Frickenhausen) (1.5 ml/well), the plates were then incubated at 37 °C and 8-10 % CO₂ for the next 10 days. For check of any abnormalities (e.g. bacterial contaminations), the fusion plates were examined visually 24-48 h after fusion and for growing of cell clusters during the next days. After 10 days, aliquots from every well with 0.5 ml were taken and tested for *C. beticola* reactive antibodies by ELISA.

2.5.5 Determination of hybridomas secreting *Cercospora*-specific antibodies

96-well microtiter plates (Sarstedt, Nümbrecht, Germany) were coated with *C. beticola* antigen (100 µl/well; 10µg protein/ml carbonate-bicarbonate buffer; 0.5 M, pH 9.6; sodium hydrogen carbonate (NaHCO₃) 2.93 g and disodium carbonate (Na₂CO₃) 1.59 g in 1 liter ddH₂O) and placed on the shaker for 1 h at room temperature. The plates were then washed two times with PBST (0.05% Tween 20 in PBS) before blocking with blocking reagent for ELISA (Roche Diagnostics, Penzberg, Germany) and incubated for 15 min at room temperature. After washing two times with PBST, the antibody-containing cell culture supernatant fluids (100µl/well) were added and the plate was placed on the shaker for 1 h. The

plate was then washed two times with PBST and 100 µl of secondary antibody was added per well (goat anti-mouse IgG, human ads-HRP, BIOZOL Diagnostica Vertrieb, Eching, Germany) and incubated for 1 h. After washing four times with PBST, BM blue POD (Tetramethylbenzidine (TMB), Roche Diagnostics, Germany) was used as substrate (100 µl/well), and the absorbance was measured at 450 nm using microplate reader machine (Magellan, Tecan Group Ltd., Männedorf, Switzerland). Clones of positive wells were picked up and transferred separately to 24 well plates. After 1 week, the clones of 24 well plates were tested again for reactivity with *C. beticola* and some other fungal species (*Chaetomium sp.*, *F. aciformis*, *F. sporotrichioides*, *P. infestans*, *R. solani*). The positive clones were transferred into Petri dishes and cultivated to produce enough amounts of antibodies and then frozen until being used for detection of *C. beticola*.

2.5.6 Using the produced monoclonal antibodies for detection and quantification of *C. beticola* antigen in soil samples

One g of the soil sample was added to 20 ml carbonate-bicarbonate buffer 0.5 M in 50 ml tube. The tube was mixed well and then homogenized three times (DIGITAL Sonifier® UNITS Models S-250D, Branson). Each cycle of homogenization was performed on ice twice at 30 sec intervals. The mixture was then centrifuged for 20 min at 10,000 g and 4 °C. The sediment was discarded and the supernatant was transferred to a clean 2 ml tube, and then it was ready to use for ELISA application as described above.

For the quantification of *C. beticola* antigen in soil samples, 0.1 g mycelium of isolate Cb12 was added to 0.9 g autoclaved field soil and ground in liquid nitrogen. The mycelium soil mix was then added to 20 ml carbonate-bicarbonate buffer in a 50 ml tube. The protocol was followed as described above until getting the supernatant of the mycelium soil mix. Different dilutions were then prepared from the supernatant (1:10 - 1:10,000) with carbonate-bicarbonate buffer which were applied at each time of ELISA application. The standard curve was prepared by the linear relationship between the OD values and the concentrations of *C. beticola* mycelium.

2.6 Detection limit of *C. beticola* in soil probes by ELISA and PCR

2.6.1 Mycelium as inoculum source

To determine the detection limit of *C. beticola* in soil, two different inoculum sources were on focus. The first one is mycelium of *C. beticola*. In this experiment, 0.1 g mycelium of isolate Cb12 was added to 0.9 g autoclaved field soil and grinded in liquid nitrogen. This substrate was mixed well and a 10-fold dilution series from the mycelium soil mix was prepared (1:10-1:10,000,000). The samples were then kept at -20°C until use for PCR and ELISA analyses.

2.6.2 Infected sugar beet leaves as inoculum source

2.6.2.1 Artificial infection of sugar beet plants

The susceptible sugar beet cultivar Klarina (KWS) was used in this experiment under the same climate chamber conditions as described for the pathogenicity test. Two months old sugar beet plants were inoculated with a spore suspension mix of three *C. beticola* isolates (Cb2, Cb3, Cb12) using an atomizer such that both sides of each leaf were wetted until run off. Subsequently, inoculated plants were incubated by covering with polyethylene bags for 3 days. Two weeks later, the symptoms of CLS had appeared on the infected leaves which were cut and kept at 4°C until use.

2.6.2.2 Preparation of infected leaves soil mix

The infected sugar beet leaves were cut into small pieces using clean scissors. These pieces were then mixed with autoclaved field soil at 1:1 ratio to make the basic mix. Different dilutions were prepared from the basic mix (1:10 - 1:1,000,000) through the addition of respective soil amounts. Each dilution was then watered and dried before being stored at -20°C for further analyses by PCR and ELISA.

2.7 Determination of inoculum degradation in soil in climate chamber using real-time PCR and ELISA

The first and second dilutions from the infected leaves soil basic mix (1:10 and 1:100, respectively, as described in section 2.6.2.2) were used in this experiment to determine the degradation of *C. beticola* inoculum in soil during 6 months. Each dilution was placed into 10 cm diameter pots and planted with one seed of the sugar beet cultivar Klarina. Three replicates from each dilution were exposed to standard climate chamber conditions (20°C, 50% rH and 14-16 h photoperiod). Three pots were filled with sterile autoclaved soil as a negative control. The soil samples were taken from each treatment at 1 month intervals and stored at -20°C until being used for ELISA and real-time PCR analyses.

2.8 Application of inoculum detection methods in the field

After developing and proving the detection methods under controlled conditions at known inoculum amounts, these methods were applied to soil samples from natural fields of different locations. The soil samples were collected from two regions, Lower Bavaria and Lower Saxony. Additionally, one location in Montana (USA, the United States Department of Agriculture) was under screen. Overall, the samples included different crop rotations and soil maintenance (Tables 5-8). The samples were taken, using a 1 m pipe tube, from three different soil layers: 0-5 cm (L1), 5-15 cm (L2) and 15-30 cm (L3). Samples were collected in May 2010 from the German fields (Bavaria and Lower Saxony) and in September 2010 from the US fields (Montana). Also, three samples were collected from a field which had never been cultivated with sugar beet before, from Schleswig-Holstein. The samples were randomly collected from each field, kept in ice boxes and then stored at -20°C until being used for ELISA and PCR analyses.

To determine the degradation of *C. beticola* inoculum in field soil, different samples were collected in June 2011, 1 year after the first survey in May 2010, from the field which contained the biggest inoculum of *C. beticola*, field number 4 of Bavaria location. The samples were collected from the same area and in the same way as done in the first survey, 2010. Each layer of soil included four repetitions which contained five single probes each and which were mixed together from an area of about 1 m².

Table 5: Crop rotation and soil maintenance of Bavarian fields (Lower Bavaria)

Year	Crop rotation and Soil maintenance	Field 1	Field 2	Field 3	Field 4
2010	Crop	Maize	Maize	Sugar beet	Winter wheat
2010	Soil maintenance	Plough	Cultivator	Plough	Cultivator
2009	Crop	Winter wheat	Sugar beet	Maize	Sugar beet
2009	Soil maintenance	Cultivator	Plough/Mustard (minimum tillage)	Plough	Plough
2008	Crop	Sugar beet	Winter barley	Winter wheat	Maize
2008	Soil maintenance	Plough	Plough	Cultivator	Plough/Mustard (minimum tillage)
2007	Crop	pea	Winter wheat	Sugar beet	Winter wheat
2007	Soil maintenance	Cultivator	Cultivator	Plough/Mustard (minimum tillage)	Cultivator
2006	Crop	-----	Sugar beet	Winter wheat	Sugar beet
2006	Soil maintenance	-----	Plough	Cultivator	Plough

Table 6: Crop rotations and soil maintenance of Lower Saxonian fields (A)

Year	Crop rotation and Soil maintenance	Field 1	Field 2	Field 3
2010	Crop	Sugar beet	Winter wheat	Winter wheat
2010	Soil maintenance	Cultivator	Plough	Cultivator
2009	Crop	Winter wheat	Winter wheat	Sugar beet
2009	Soil maintenance	Plough	Cultivator	Cultivator
2008	Crop	Winter wheat	Sugar beet	Winter wheat
2008	Soil maintenance	Cultivator	Cultivator	Plough
2007	Crop	Sugar beet	Winter wheat	Winter wheat
2007	Soil maintenance	Cultivator	Plough	Cultivator
2006	Crop	Winter wheat	Winter wheat	Rape
2006	Soil maintenance	Plough	Cultivator	Plough

Table 7: Crop rotations and soil maintenance of Lower Saxonian fields (B)

Year	Crop rotation and Soil maintenance	Field 4	Field 5	Field 6
2010	Crop	Winter wheat	Winter wheat	Sugar beet
2010	Soil maintenance	Cultivator	Cultivator	Plough
2009	Crop	Rape	Sugar beet	Winter wheat
2009	Soil maintenance	Plough	Plough	Cultivator
2008	Crop	Winter wheat	Winter wheat	Winter wheat
2008	Soil maintenance	Cultivator	Cultivator	Cultivator
2007	Crop	Sugar beet	Winter wheat	Sugar beet
2007	Soil maintenance	Cultivator	Cultivator	Plough

Table 8: Crop rotations and soil maintenance of US fields (Montana, USDA)

Year	Crop rotation and Soil maintenance	Field 1	Field 2
2010	Crop	Sugar beet	Soybean
2010	Soil maintenance	Conventional tillage	No-till
2009	Crop	Barley	Barley
2009	Soil maintenance	Conventional tillage	Conventional tillage
2008	Crop	Sugar beet	Sugar beet
2008	Soil maintenance	Conventional tillage	Strip tillage
2007	Crop	Barley	Barley
2007	Soil maintenance	Conventional tillage	Conventional tillage
2006	Crop	Sugar beet	Sugar beet
2006	Soil maintenance	Conventional tillage	Strip tillage

3 Results

3.1 Pathogenicity test

Pathogenicity tests were carried out under standard conditions in climate chamber at 25 to 22° C (day/night, respectively), 14-16 h photoperiod (following the method of Shane and Teng 1983) and 50% rH.

These tests were carried out for comparison between different isolates of *C. beticola* depending on their Pathogenicity. Symptoms appeared on inoculated leaves 6-7 days after inoculation. Pinpoint, reddish purple spots later enlarged to angular-to-irregular lesions, which led to chlorosis and necrosis of leaf tissue.

The records of symptom appearance revealed that all isolates were pathogenic on the susceptible sugar beet cultivar Klarina (Table 9). Disease Severity ranged from 24.1 to 2 % within the three isolate groups, depending on the location, where the German isolates were the highest pathogenic. *C. beticola* isolate Cb2 showed the highest disease severity for the plants. The Egyptian *C. beticola* isolates were the lowest pathogenic for the plants and E2 showed the lowest disease severity.

Table 9: Pathogenicity of *Cercospora beticola* isolates

Isolate	Origin	Disease Incidence (%)	Disease Severity (%)
Cb	Germany	2	15.6
Cb1	Germany	1.8	12
Cb2	Germany	2.5	24.1
Cb3	Germany	1.8	15.6
Cb4	Germany	0.6	4.5
Cb5	Germany	1.6	8.3
Cb6	Germany	1.3	12.1
Cb7	Germany	0.5	6.5
Cb9	Germany	0.75	4.8
Cb10	Germany	2	15
Cb12	Germany	1.8	12
Cb13	Germany	1.16	9
E1	Egypt	0.27	3
E2	Egypt	1.3	2
E3	Egypt	0.27	3
E4	Egypt	0.36	4
C1	USA	5	10
C2	USA	4.5	10
S1	USA	0.8	5.3
S2	USA	2.5	4.5

3.2 Design of specific primers for *C. beticola*

3.2.1 Search of *C. beticola*- specific sequences in NCBI-GenBank

Three primer sets were designed using the internet program primer3 (<http://frodo.wi.mit.edu/primer3/input.htm>) after definition of three sequences of *C. beticola* in the NCBI-GenBank ([http:// www.ncbi.nlm.nih.gov/](http://www.ncbi.nlm.nih.gov/)): a 469-bp rDNA sequence

(AY343371), a 967-bp cytochrome b (cytb) mRNA sequence (EF176921) and a 1195-bp actin gene (AF443281), (Fig. 10a-c).

A sequence of rRNA (496 bp)
 GGATCATTACTGAGTGAGGGCCTTCGGGCTCGACCTCCAACCCTTTGTGAACACAACCTTGTTGCTTCGGG
 GGCGACCCTGCCGTTTCGACGGCGAGCGCCCCGGAGGCCTTCAAACACTGCATCTTTGGCTCGGAGTTT
 AAGTAAATTAACAAAACTTTCAACAACGGATCTCTTGGTTCGGCATCGATGAAGAACGCAGCGAAATG
 CGATAAGTAATGTGAATTGCAGAAATCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCCTGGTA
 TTCCGAGGGGCATGCCTGTTGAGCGTCATTTCAACACTCAAGCCTCGCTTGGTATTGGGCGCCGCGGTG
 TTCCGCGCGCCTCAAAGTCTCCGGCTGAGCTGTCGGTCTCTAAGCGTTGTGATTTCAATTAATCGCTTCGG
 AGCGCGGGCGGTTCGGCCGTTAAATCTTTACAAGGTTGACCTCGGAT

Fig. 10a: The sequence of rRNA of *Cercospora beticola*. The sequence of designed primer is underlined.

A sequence of cytochrome b (mRNA) (967 bp)
 AGAGCATATGATGAGAGACGTAAATAACGGATGATTAATACGTTACTTACACTCTAATACAGCTTCAGCA
 TTTTTCTTCTTAGTATATTTACACGTAGGAAGAGGTCTATACTATGGTTCCTTACAAAGCACCTAGAACAT
 TGGTATGAACTATAGGTACTATTATATTAGTTTTAATGATGGCTACAGCCTTCTTGGGTTATGTTTTACC
 TTACGGACAAATGTCTTTATGAGGTGCAACTGTTATTACTAATTTAATGAGTGCAATACCATGAGTAGGA
 CAAGACATAGTTGAGTTTTTATGAGGAGTTTTTCTGTTAATAACGCAACATTAATAATAGATTTTTTGCAT
 TGCATTTTGTATTACCTTTCGTATTAGCTGCATTAGCTTTAATGCACCTAATTGCTTTACATGATAGTGC
 AGGTTACAGGTAATCCTTTAGGAGTTTTCTGGTAATTATGACAGACTTCTTTTTGCTCCATACTTTATATTT
 AAAGATTTAATAACTATATTTCTTATTTATAATAGTGCTATCAGTGTGTTTTCTTTCATGCCGAACGTTT
 TAGGTGATAGTGAGAATTACGTTGTGGCCAATCCAATGCAAACACCTCCTGCTATAGTACCAGAATGATA
 TTTATTACCTTCTATGCTATATTAAGATCTATACCTAACAACTATTAGGTGTTATTGCTATGTTTTCT
 GCCATATTAATAACTAATACTAATGCTTTTACAGACCTAGGTAGAAGTAGAGGATTACAGTTCAGACCTT
 TAAGTAAAATAGCATTTTACATTTTGTAGCAAATTTCTTATTATTGATGCAATTGGGTGCTAAACACGT
 TGAATCACCATTATAGAATCTAGTCAAATAAGTACTGTTTTATATTTTTTACATTTCTTGATAATAGTT
 CCTTTAGTTAGTTTATTAGAAAATAGTTTAAATTGAATTACATTTAAATAAAAAATAA

Fig. 10b: The sequence of cytochrome b of *Cercospora beticola*. The sequence of designed primer is underlined.

A sequence of actin gene (1195 bp)
 ACTCATGGCCGGTTTGTATGTGCAAGGCCGGTTTGTATGTGCAAGGCCGGTTTCGCCGGTGACGATGCGC
 CACGAGCTGTCTCCGTAAGTGCTGCCACAATCAGACGCCAAAAGCTGGCAGGAAGGAGGAGCTGACATTG
 GGACAGCATCCATCCATCGCTCGGACGACCCGCGCCACCATGGGTATGCGATCCGCCGTCTCCGCCGTGCAAAAT
 CCTCCTAACAAGAGCAGATCATGATTGGTATGGTCAGAAGGACTCATATGTCGGTGATGAGGCACA
 GAGCAAGCGTGGTATCCTGACGCTGAGATACCCCATCGACACGGTGTATCACCAACTGGGACGACATG
 GAGAAGATCTGGCACCACACCTTCTACAACGAGCTCCGTGTGCGCACCAGAGGAGCACCTGTCTGCTCA
 CCGAGGCTCCAATCAACCCAAAGTCCAACCGTGAGAAGATGACACAGATTGTCTTCGAGACGTTCAACGC
 ACCAGCCTTCTACGTCTCCATCCAGGCCGCTCTTTCCCTGTACGCTTCCGGTCTGACCCGGTATCGTG
 CTCGACTCCGGTGACGGAGTTACCCACGTTGTCCCATCTACGAGGGTGTGCTCTCCACACGCCATCT
 CCCGTGTGACATGGCTGGTTCGTGATTTGACCGACTACCTCATGAAGATCTTGGCTGAGCGCGGATACGT
 TTTCTCCACCACCGCCGAGCGTGAAATCGTTTCGTGACATCAAGGAGAAGCTCTGCTACGTCGCCCTCGAC
 TTCGAGCAGGAAATTCAAACCGCCAGCCAGAGCTCTTCGCTCGAGAAGTCTACGAGCTTCTGACGGAC
 AGGTCATCACCATCGGCAACGAGCGTTTTCCGTGCACCAGAGGCCCTCTCCAGCCATCCGTCTCGGTCT
 CGAATCTGGCGGTATCCACGTCACCACCTTCAACTCCATCATGAAGTGTGATGTCGATGTCCGCAAGGAT
 CTCTACGGAAACATCGTCATGGTAAGCTGGCCCCATCATTTATGATCTGGAAGAGATTGAGCTGACAAAT
 TTTAGTCTGGTGGCACCACCATGTACCCAGGTATCTCCGACCGTATGCAAAAAGGAAATCACCGCCTTGGC
 CCCATCCAGCATGAAGGTCAAGATCATCGCACCACCGGAGCGCAAGTACTCCGTCTGGATCAGTGGTTCA
 AGCTT

Fig. 10c: The sequence of actin gene of *Cercospora beticola*. The sequence of designed primer is underlined.

The characters of the three designed primer sets are shown in Table 10. These primers have different GC content, but have similar melting temperature (T_m). The 3' self-complementarity score was small for all primers which indicate that these primers have low capability to form a primer dimer. The self-complementarity score (Any) was different in the three primer sets and the highest self-complementarity scores were obtained from the primer pairs Cb-actinF1 and Cb-actinR2.

Table 10: Characters of the designed primer sets and their product sizes

Primer	Primer length	T_m	GC%	Any	3'	Product size
Cb-actinF1	24	60.16	41.67	8.00	2.00	798-bp
Cb-actinR2	19	59.91	52.63	4.00	2.00	
Cyt-F	20	58.98	45.00	4.00	0.00	243-bp
Cyt-R	20	59.38	40.00	6.00	2.00	
ITS3	20	60.26	50.00	6.00	0.00	223-bp
ITS4	20	60.25	50.00	3.00	2.00	

The values of T_m , Any and 3' in this table were obtained from the internet programme Primer3 when these primers were designed. Any means the self-complementarity score of the primer (taken as a measure of its tendency to anneal to itself or form secondary structure). 3' means the 3' self-complementarity of the primer (taken as a measure of its tendency to form a primer-dimer with itself).

3.2.2 The PCR amplification primary tests for the designed primer sets

The three primer sets, Cb-actinF1 (5'-AGCACAGTATCATGATTGGTATGG-3')/Cb-actinR2 (5'-AATGATGGGGCCAGCTTAC-3'), Cyt-F (5'-CAATCCAATGCAAACACCTC-3')/Cyt-R (5'-GCACCCAATTGCATCAATAA-3') and ITS3 (5'-AGGCCTTCAAACACTGCATC-3')/ITS4 (5'-CGAGGCTTGAGTGGTGAAAT-3'), were primarily tested with three *C. beticola* isolates; Cb2, Cb8, Cb12 (Fig. 11). The results showed that the three primers sets amplified one band from the *C. beticola* isolates with different sizes; 798-bp for Cb-actinF1/Cb-actinR2, 243-bp for Cyt-F/ Cyt-R and 223-bp for ITS3/ ITS4.

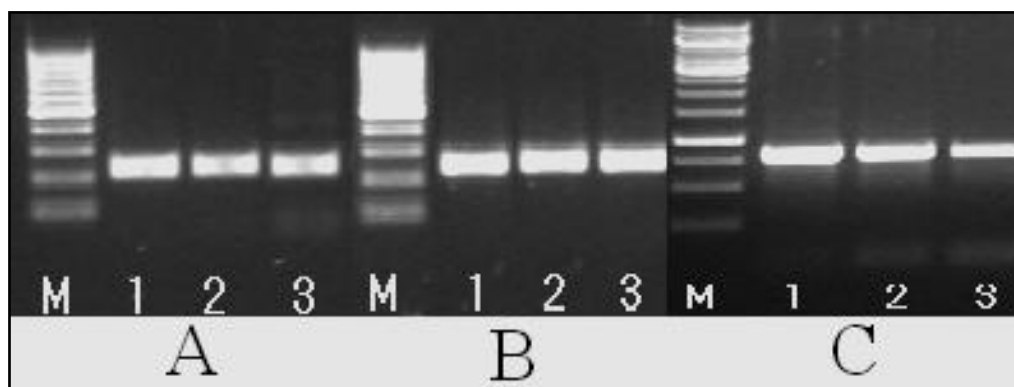


Fig. 11: The PCR amplification of the three designed primer sets; Cb-actinF1/ Cb-actinR2 (A), Cyt-F/ Cyt-R (B) and ITS3/ ITS4 (C). Lanes 1,2 and 3 are Cb2, Cb8 and Cb12, respectively. M in A and B is a 100-bp marker, M in C is a 1000-bp marker.

3.2.3 The specificity tests of the designed primer sets

The specificity tests were carried out for the three designed primer sets; Cb-actinF1/ Cb-actinR2, Cyt-F/ Cyt-R and ITS3/ ITS4, using the genomic DNA of *C. beticola* (25 isolates from Germany, Egypt and USA) and 28 other fungal pathogens.

The results showed that the two primer sets Cb-actinF1/ Cb-actinR2 and ITS3/ ITS4 amplified one single fragment, with 798-bp and 223-bp, respectively, only from *C. beticola* but not from the other tested fungi (Fig. 12 A-B and E-F, respectively). The primer set Cyt-F/ Cyt-R also established a high specificity; exceptionally from *F. oxysporum* f.sp. *vasinfectum*, one slight band was amplified (Fig 12 C and D).

Cercospora beticola is considered to be a genetically highly diverse species (Groenewald *et al.* 2008), therefore, if the primer sets developed using one isolate are located in genetic diverse regions, they may fail to amplify products from other isolates with different sequences. Accordingly, 25 *C. beticola* isolates from Germany, Egypt and USA were tested using the primer sets Cb-actinF1/ Cb-actinR2 and ITS3/ ITS4 by conventional PCR. The results (Fig. 13) showed that the developed primer sets ITS3/ ITS4 amplified one fragment from all *C. beticola* isolates tested. However, the developed primer sets Cb-actinF1/ Cb-actinR2 amplified one strong fragment from 24 *C. beticola* isolates, while one isolate, C2 (from USA), showed one slight fragment.

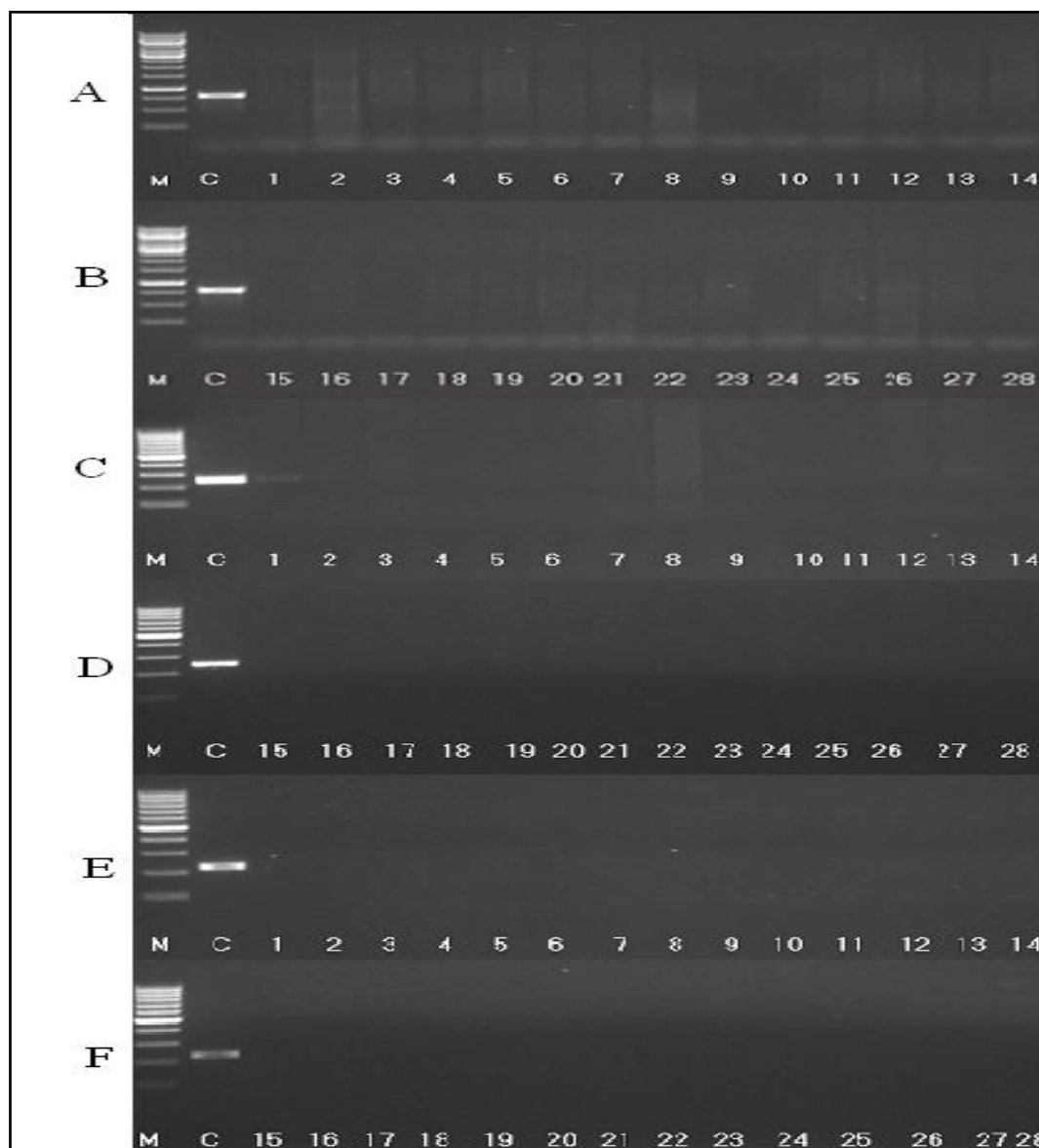


Fig. 12: The specificity tests of the primers Cb-actinF1/ Cb-actinR2 (A and B), Cyt-F/ Cyt-R (C and D) and ITS3/ ITS4 (E and F) by PCR with genomic DNA of *Cercospora beticola* and 28 fungal pathogens. M in A and B is a 1000 bp marker, M in C, D, E and F is a 100 bp a marker. C is *C. beticola*, the lanes 1 to 28 are *F. oxysporum* f.sp. *vasinfectum* (1), *F. moniliforme* (2), *F. avenaceum* Rg 7/5 (3), *Fusarium solani* (4), *F. sporotrichioides* (5), *F. graminearum* 37 (6), *F. antheophilus* (7), *F. culmorum* (8), *F. tricinctum* (9), *Rhizoctonia solani* (AG2-1) (10), *Phytophthora ramorum* (11), *P. infestans* (12), *Macrophomina phaseolina* (13), *Chaetomium* spp. (14), *Verticillium dahlia* (15), *Septoria petroselini* (16), *Microdochium nivale* (17), *Stagonospora nodorum* (18), *Gliocladium catenulanum* (19), *Pseudocercospora anguioides* (20), *Rhizopus* spp. (21), *Pyrenophora graminea* 15 (22), *Botrytis* spp. (23), *Cladosporium* spp. (24), *Alternaria alternate* (25), *Pseudocercospora herpotrichoides* var. *acufomis* (26), *Cyathus striatus* (27) and *C. olla* (28).

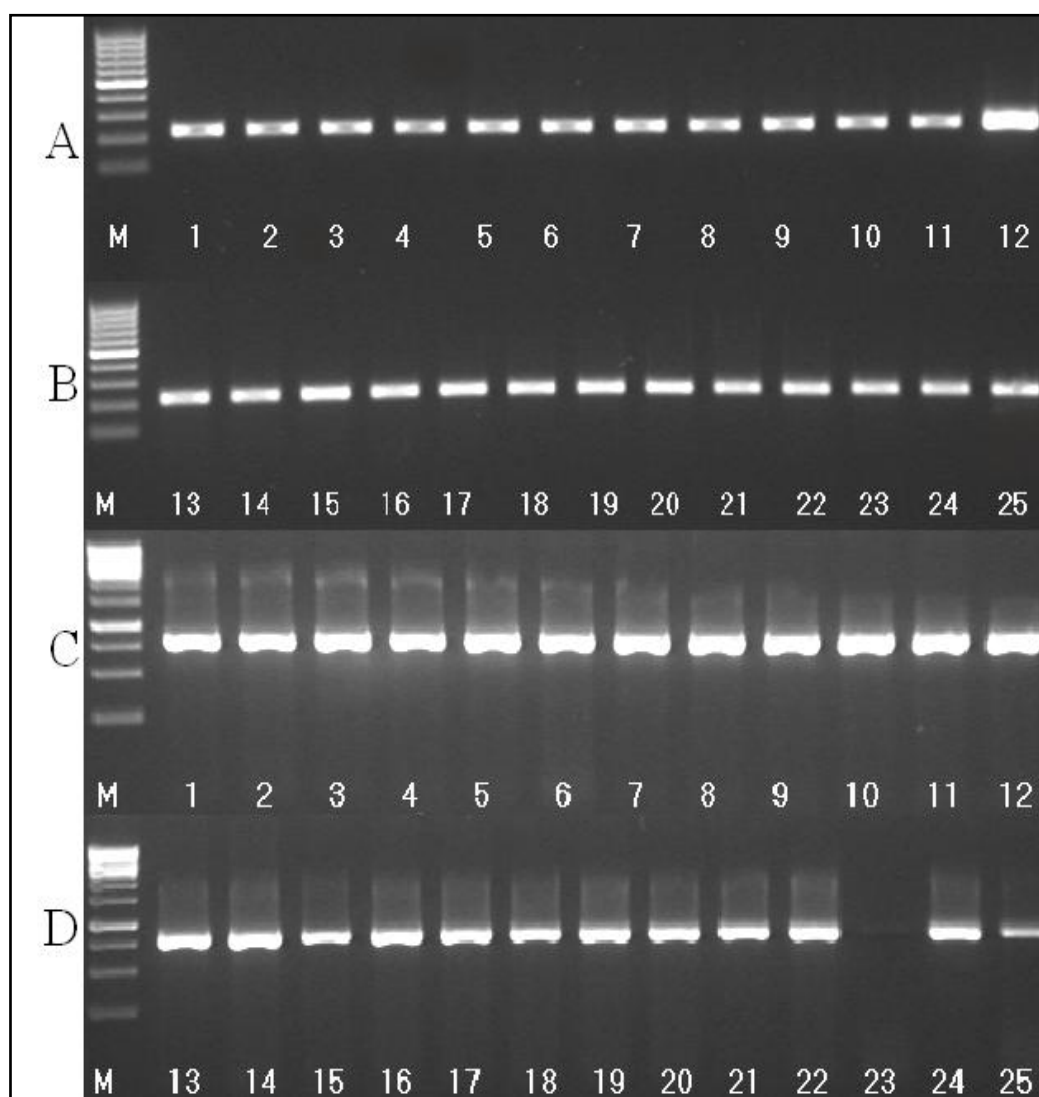


Fig. 13: The PCR amplification of genomic DNA of 25 *Cercospora beticola* isolates from different countries using the primer pairs ITS3/ ITS4 (A and B) and Cb-actinF1/ Cb-actinR2 (C and D). M in A and B is a 100 bp marker, M in C and D is a 1000 bp marker. The lanes 1 to 25 are isolates Cb, Cb1, Cb2, Cb3, Cb4, Cb5, Cb6, Cb7, Cb9, Cb10, Cb12, Cb13, CbE1, CbE2, CbE3, CbE4, CbE5, CbE6, CbE7, CbE8, CbE9, C1, C2, S1, S2, respectively.

3.2.4 The sensitivity tests of two specific primer sets

The detection sensitivity is an important element for the diagnostic application of a primer set. The sensitivity tests of two primer sets; Cb-actinF1/ Cb-actinR2 and ITS3/ ITS4, were carried out using 10 different concentrations of genomic DNA of *C. beticola*, 100 ng, 10 ng, 1 ng, 100 pg, 50 pg, 10 pg, 5 pg, 1 pg, 0.5 pg, and 0.1 pg. Three different cycle numbers of conventional PCR were used, 28, 35 and 40 cycles. Ten μ l of PCR products were visualized on 1.7 % agarose gel stained with ethidium bromide.

At 28 cycles, the primer set ITS3/ ITS4 showed that it can detect as little as 100 pg of *C. beticola* genomic DNA (Fig. 14A), but the primer set Cb-actinF1/ Cb-actinR2 had much lower detection sensitivity (Fig. 14B), it can detect only up to 1 ng. At 35 cycles, the primer set ITS3/ ITS4 showed that it can detect as little as 5 pg of *C. beticola* genomic DNA (Fig. 14C), but the primer set Cb-actinF1/ Cb-actinR2 had much lower detection sensitivity (Fig. 14D), it can detect only up to 100 pg. At 40 cycles, the primer set ITS3/ ITS4 showed a highest level of detection, it can detect as little as 0.5 pg (Fig. 14E), while the primer set Cb-actinF1/ Cb-actinR2 can only detect up to 10 pg of *C. beticola* genomic DNA (Fig. 14F). From the previous results, it is clear that the primer set ITS3/ ITS4 has a higher level of detection than the primer set Cb-actinF1/ Cb-actinR2.

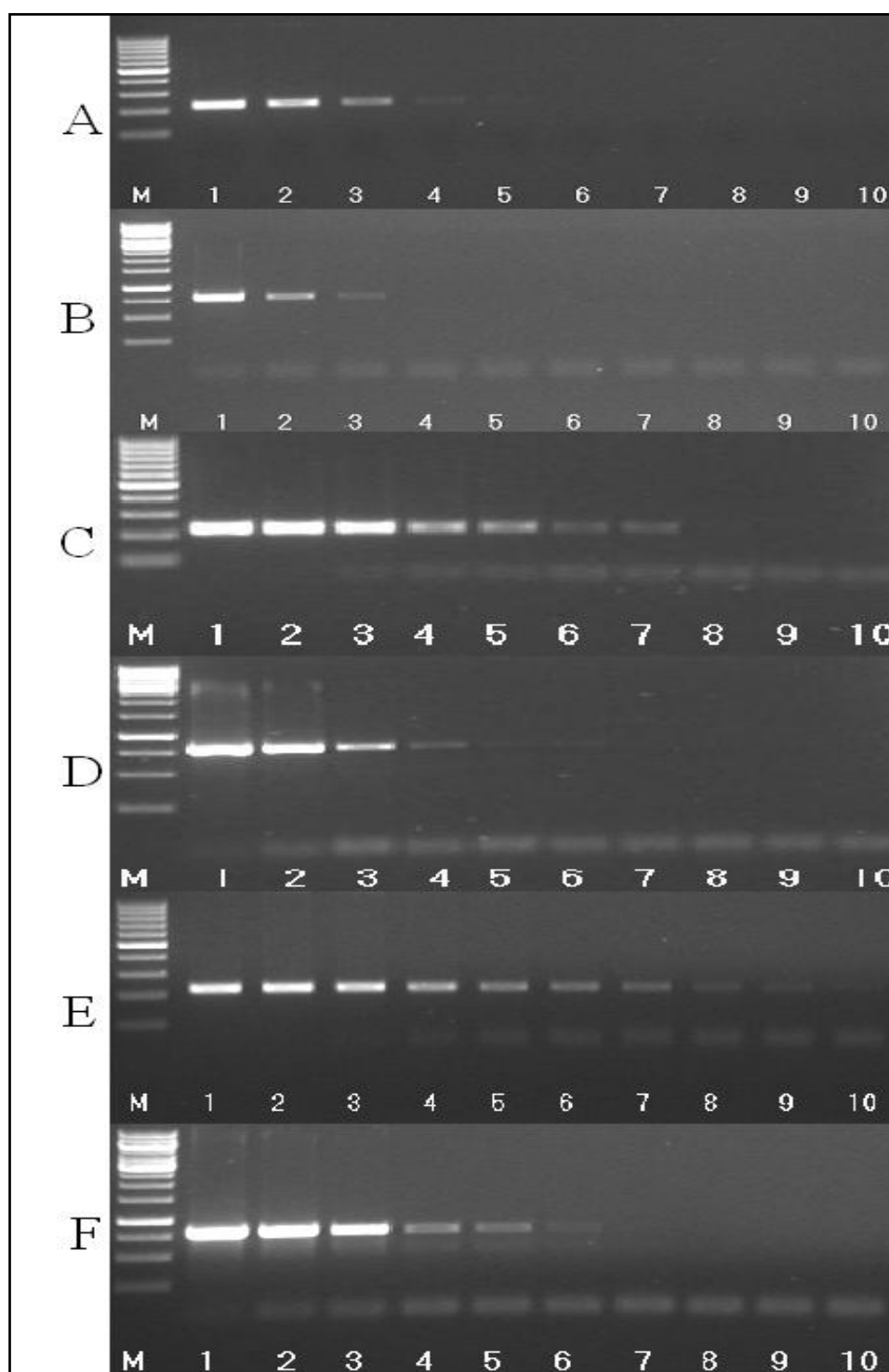


Fig. 14: The sensitivity tests of two primer sets ITS3/ ITS4 (A, C and E) and Cb-actinF1/ Cb-actinR2 (B, D and F) at 28 cycles (A and B), 35 cycles (C and D) and 40 cycles (E and F) of conventional PCR. M in A, C and E is a 100 bp marker and M in B, D and F is a 1000 bp marker. The lanes 1 to 10 are *Cercospora beticola* genomic DNA 100 ng, 10 ng, 1 ng, 100 pg, 50 pg, 10 pg, 5 pg, 1 pg, 0.5 pg, and 0.1 pg, respectively.

3.3 Monoclonal antibody production and their specificity test

The method of Köhler and Milstein (1975) was used to produce monoclonal antibodies against cell wall of *C. beticola* as described in materials and methods (Section 2.5). The hybridomas clones, which resulting from fusion of antibody-producing B-cells and myeloma cells, were tested against *C. beticola*. The clones of positive wells were picked up and transferred separately to 24 well plates. Eighteen positive clones were obtained against *C. beticola* which were tested after 1 week again for reactivity with *C. beticola* and some other fungal species; *Chaetomium sp.*, *Fusarium aciformis*, *F. sporotrichioides*, *Phytophthora infestans* and *Rhizoctonia solani*.

The results showed that all antibody clones gave positive OD values only with *C. beticola* and negative OD values with the other fungal species indicating that these antibodies are specific for *C. beticola* (Table 11). OD values (Optical density or the absorbance) taken as a measure of the antigen concentration in sample, where it has a positive relationship with the antigen concentration. The results showed that highest OD value with *C. beticola* (2.7) was obtained from clone C103, while the clone C209 gave the lowest OD value (0.18).

Table 11: OD-values after specificity test of antibodies (clones of monoclonal antibodies) towards antigens of *Cercospora beticola* and some other fungal species

Clones	<i>Cercospora beticola</i>	<i>Chaetomium sp.</i>	<i>Fusarium oxysporum</i>	<i>Phytophthora infestans</i>	<i>Fusarium sporotrichioides</i>	<i>Rhizoctonia solani</i>
C100	1.50*	0.06	0.06	0.06	0.06	0.06
C101	1.60	0.06	0.06	0.06	0.06	0.06
C102	0.70	0.06	0.06	0.06	0.06	0.06
C103	2.70	0.06	0.06	0.06	0.06	0.06
C104	2.30	0.06	0.06	0.06	0.06	0.06
C105	1.90	0.06	0.06	0.06	0.06	0.06
C106	2.30	0.06	0.06	0.06	0.06	0.06
C107	1.90	0.06	0.06	0.06	0.06	0.06
C201	2.44	0.02	0.02	0.02	0.02	0.02
C202	2.53	0.02	0.02	0.02	0.02	0.02
C203	0.69	0.02	0.02	0.02	0.02	0.02
C204	1.13	0.02	0.02	0.02	0.02	0.02
C205	0.19	0.02	0.02	0.02	0.02	0.02
C206	0.17	0.02	0.02	0.02	0.02	0.02
C207	1.01	0.02	0.02	0.02	0.02	0.02
C208	0.78	0.02	0.02	0.02	0.02	0.02
C209	0.18	0.02	0.02	0.02	0.02	0.02
C210	1.03	0.02	0.02	0.02	0.02	0.02

3.4 Determination of detection limit in soil probes by ELISA and PCR

This experiment was carried out to define the limit of developed antibodies (mAbs) and primer sets, ITS3/ ITS4 and Cb-actinF1/ Cb-actinR2, for *C. beticola* detection in soil. For this purpose, two different inoculum sources were on focus, *C. beticola* mycelium and *C. beticola*-inoculated sugar beet leaves.

3.4.1 Detection limit of *C. beticola* in soil probes using PCR

3.4.1.1 *C. beticola* mycelium as soil inoculum

0.1 g of *C. beticola* mycelium, isolate Cb12, was added to 0.9 g autoclaved field soil and grinded in liquid nitrogen. After mixing, a 10-fold dilution series from the mycelium soil mix was prepared (mycelium soil mix: soil = 1:10- 1:10,000,000).

The result shows that the primer set ITS3/ ITS4 (Fig. 15A) has the highest level of detection. It can detect even the minimum dilution (1:10,000,000), while the primer set Cb-actinF1/ Cb-actinR2 (Fig. 15B) can detect until the fifth dilution only (1:10,000).

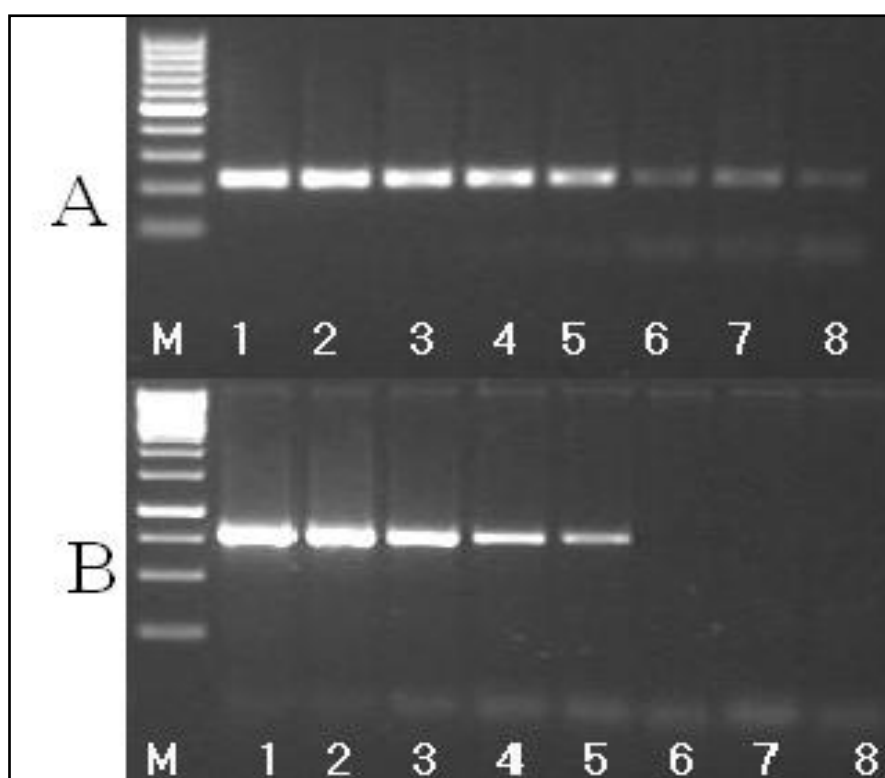


Fig. 15: Limit of two primer sets ITS3/ ITS4 (A) and Cb-actinF1/ Cb-actinR2 (B) to detect *Cercospora beticola* from infected soil using mycelium as inoculum. M in A is a 100 bp marker and M in B is a 1000 bp marker. The lanes reveal the dilutions 1-8.

3.4.1.2 *C. beticola*-infected sugar beet leaves as soil inoculum

The infected sugar beet leaves were cut into small pieces using a clean scissor, and then mixed with autoclaved field soil at 1:1 ratio to make the basic mix. Different dilutions were

prepared from the basic mix (infected leaves soil mix: soil =1:10 - 1:1,000,000) by adding adequate soil amounts.

The result shows that the primer set ITS3/ ITS4 (Fig. 16A) has the highest level of detection. It can detect the sixth dilution (1:100,000), while the primer set Cb-actinF1/ Cb-actinR2 (Fig. 16B) can detect until the fifth dilution only (1:10,000)

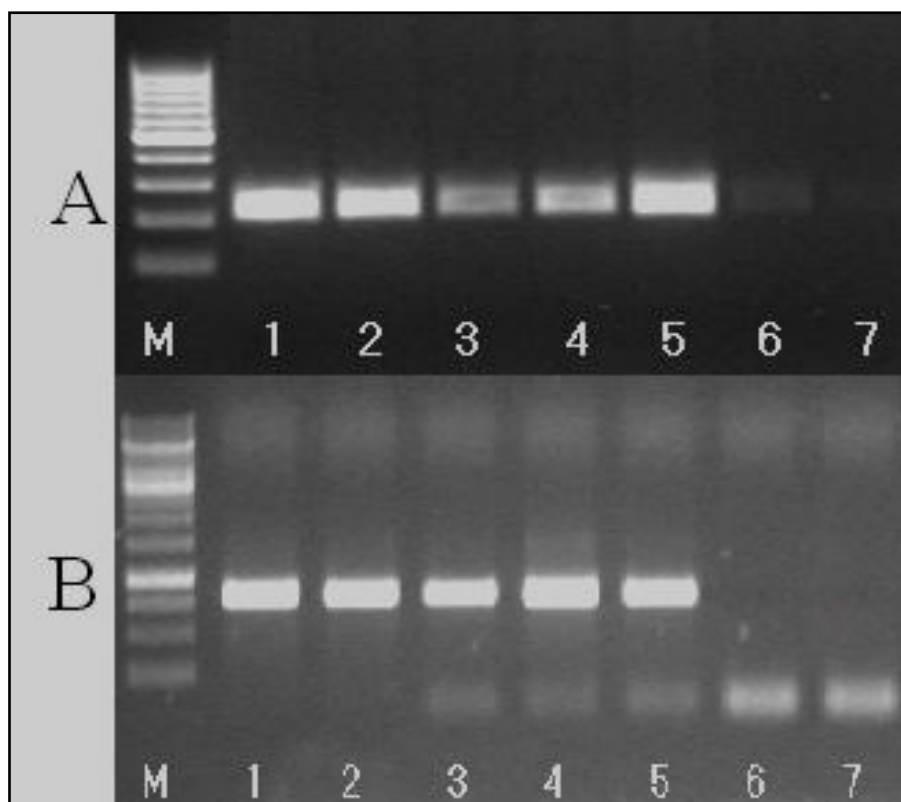


Fig. 16: Limit of two primer sets ITS3/ ITS4 (A) and Cb-actinF1/ Cb-actinR2 (B) to detect *Cercospora beticola* from infected soil using *C. beticola*-infected sugar beet leaves as inoculum. M in A is a 100 bp marker and M in B is a 1000 bp marker. The lanes reveal the dilutions 1-8.

3.4.2 Determination of detection limit in soil probes by ELISA

3.4.2.1 *C. beticola* mycelium as soil inoculum

96-well microtiter plates were coated with a dilution series from the mycelium soil mix (1:10-1:10,000,000), after extracting the antigens as described in materials and methods, and tested by ELISA assay.

The results in Table 12 show that the ELISA method can detect until the fifth dilution (mycelium soil mix: soil =1:10,000) which gave an OD value of 0.29. The OD values ranged from 2.01, the first dilution, to 0 in the sixth dilution.

Table 12: Detection limit of *C. beticola* mycelium in soil probes by using ELISA

Dilution No.	1	2	3	4	5	6	7	8
OD	2.01	0.97	0.41	0.35	0.29	0	0	0

3.4.2.2 *C. beticola*- infected sugar beet leaves as soil inoculum

Seven dilutions (infected leaves soil mix: soil =1:10 - 1:1,000,000) from the infected leaves soil mix were prepared and the extraction of antigens was carried out as described in materials and methods, and then tested by ELISA assay.

The results in Table 13 show that the ELISA can detect until the fourth dilution (1:1000) which gave an OD value of 0.18. The OD values ranged from 0.31, the first dilution, to 0 in the fifth dilution.

Table 13: Detection limit of *Cercospora beticola* in soil probes by ELISA using *C. beticola*-infected sugar beet leaves as soil inoculum

Dilution No.	1	2	3	4	5	6	7
OD	0.31	0.27	0.19	0.18	0	0	0

3.5 Determination of inoculum degradation in soil under controlled conditions in climate chamber

The objective of this experiment was to determine the degradation of *C. beticola* inoculum in soil during 6 months. The basic mixtures of soil and infected leaves (the first and second dilution, infected leaves soil mix: soil =1:10 and 1:100, respectively), were used as a sources of inoculum in soil. The temperature in climate chamber was adjusted to +20°C, 50% relative

humidity and 16 h/day light. Three pots were filled with autoclaved soil without any inoculum as a control.

3.5.1 Determination of inoculum degradation in soil using real-time PCR

The results show the quantity of *C. beticola* in soil (Fig. 17 and Table A14). The inoculum degraded in soil at both dilutions. The highest inoculum quantity was obtained always from the first dilution, where it was 781.0133 ng/g soil at the time of inoculation and degraded to 2.4726 ng/g soil after 6 months. Also, the second dilution shows degradation of inoculum over time, since the quantity of inoculum was 10.444 ng/g soil at the beginning and 0.708 ng/g soil after 6 months. Negative results were obtained from the control soil.

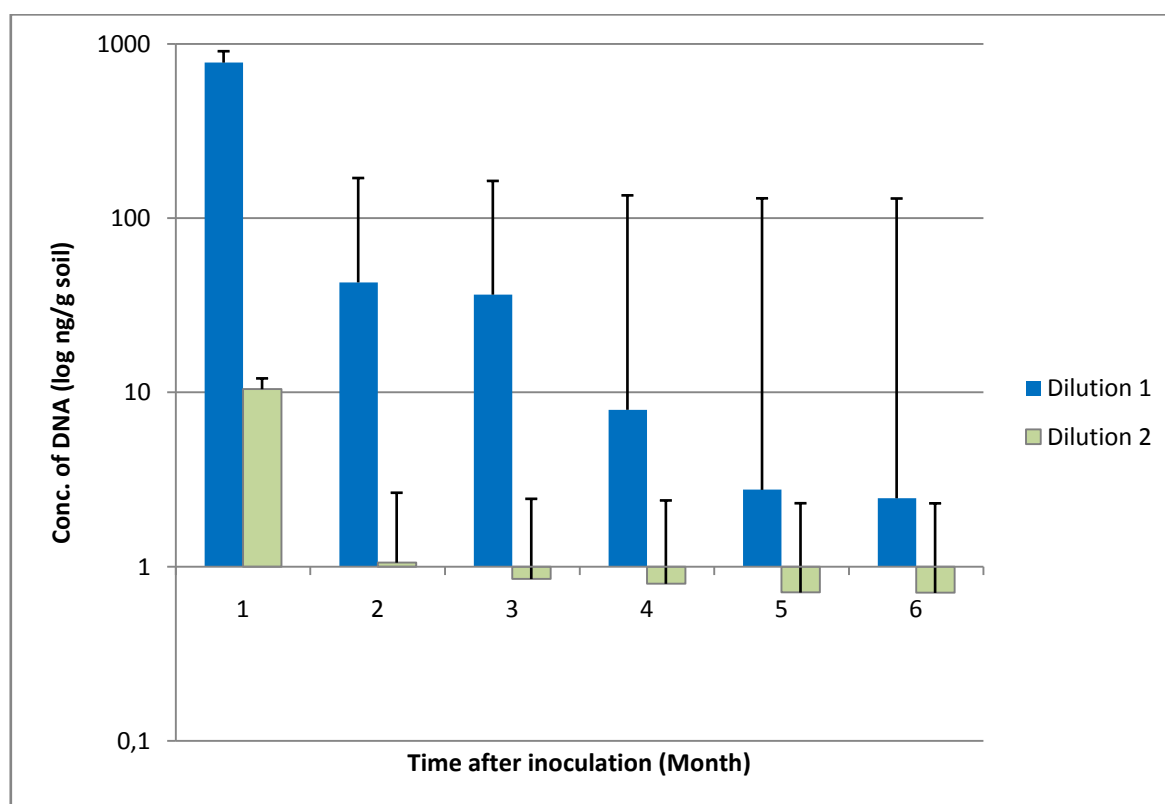


Fig. 17: Determination of *Cercospora beticola* inoculum degradation in soil by real-time PCR. Error bars indicate standard error.

3.5.2 Determination of inoculum degradation in soil using ELISA

The OD values in this experiment were converted to amount of *C. beticola* mycelium, where different concentrations of *C. beticola* mycelium soil mix were prepared by adding carbonate buffer as described in material and methods. The standard curve was then created by the linear relationship between the OD values and the concentrations of *C. beticola* mycelium. The coefficient of determination (R^2) was 0.9877 (Fig. 18) and the absorbance reading (OD value) $y = 0.0006x + 0.0294$, where $x = \mu\text{g mycelium} / \text{g soil}$.

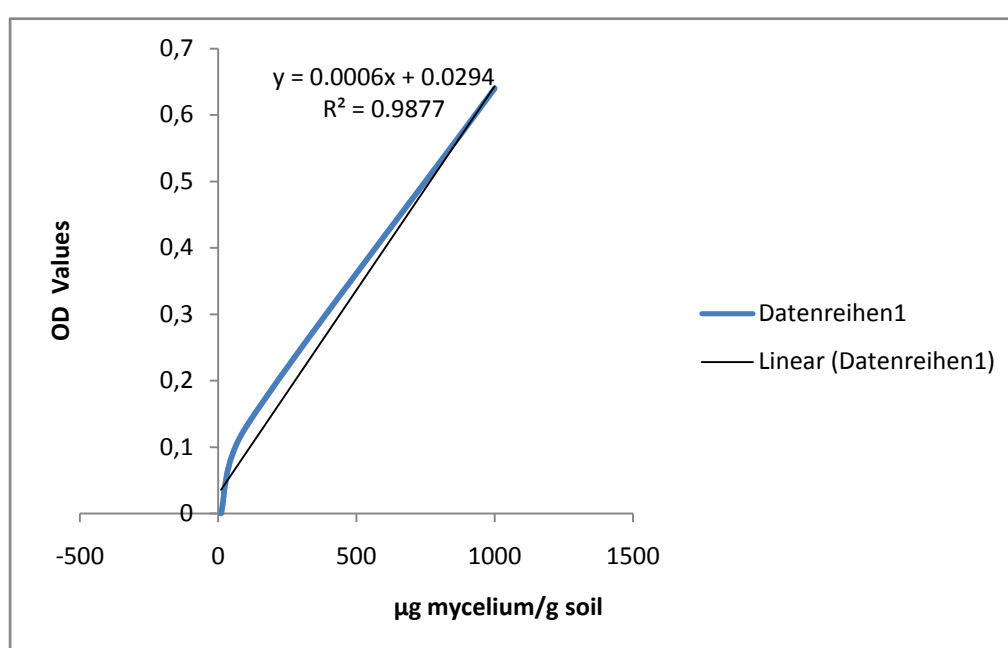


Fig. 18: The standard curve for the quantification of *Cercospora beticola* using ELISA; dependence of OD values on the amount of mycelium in soil.

The results show the time course of *C. beticola* inoculum degradation in soil (Fig. 19 and Table A28). The inoculum decreased during this 6-month study in both dilutions. The highest inoculum quantity was obtained always from the first dilution (Dilution 1). Here, the inoculum decreased from 467.66 $\mu\text{g mycelium/g soil}$ to 301 $\mu\text{g/g soil}$. Similarly, the second dilution (Dilution 2) indicated a decline of inoculum, where the quantity dropped from 401 $\mu\text{g/g soil}$ to 284.33 $\mu\text{g/g soil}$. Negative results were obtained from the control soil.

Compared with the results of real-time PCR, ELISA established slower disappearance of *C. beticola* antigen in soil than DNA degraded. The latter findings indicate that the antigen of the fungus persists in soil longer than DNA.

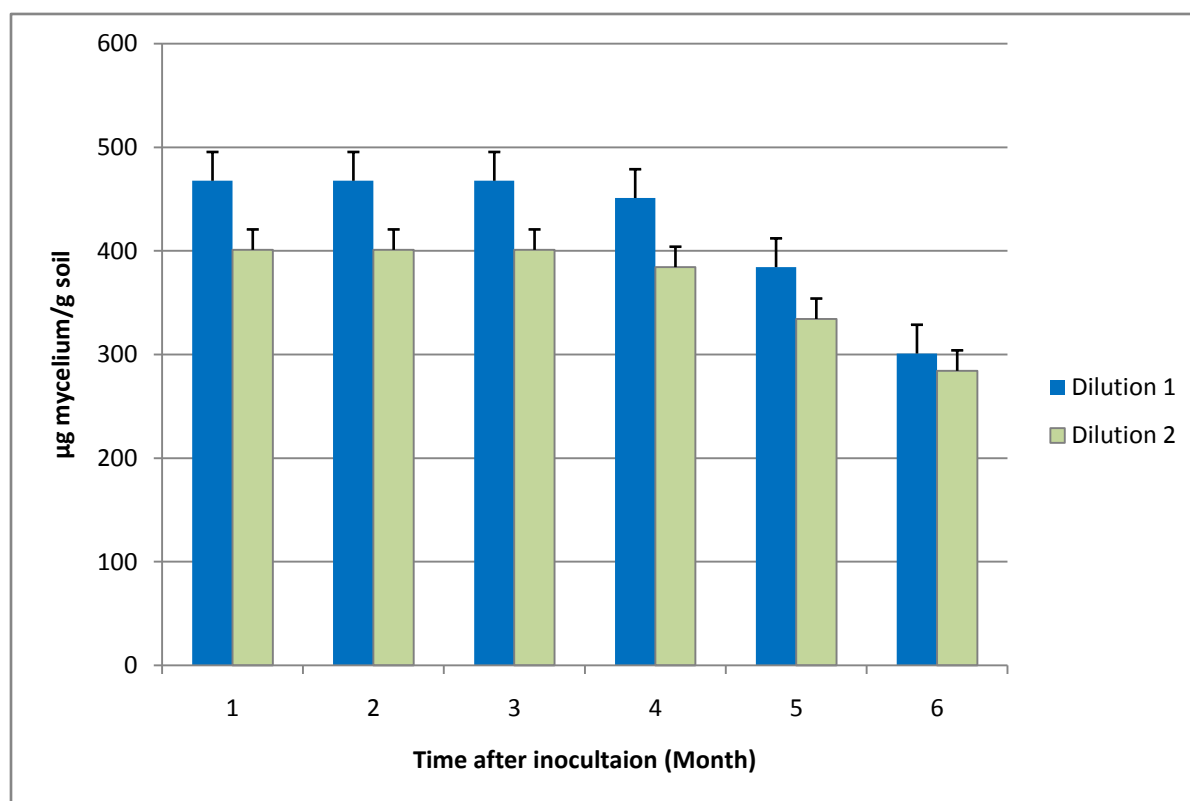


Fig. 19: Determination of *Cercospora beticola* inoculum degradation in soil by ELISA. Error bars indicate standard error.

3.6 Application of inoculum detection methods in the field

The soil samples were collected from two locations in Germany, Bavaria (4 fields) and Lower Saxony (6 fields). Additionally one location in Montana, USA (2 fields) was investigated. From each field three different layers were analyzed, 0-5 cm, 5-15 cm and 15-30 cm. To elucidate the influence of cropping measures on the inoculum situation in soil, fields with varying constellation of crop rotation and soil maintenance were surveyed. Additionally, three samples were collected from one field where sugar beet had never been cultivated before. The results of these three samples obtained by real-time PCR and ELISA were always negative.

3.6.1 Quantification of *C. beticola* inoculum using real-time PCR

The soil DNA of the different fields was tested firstly with the primer set ITS3/ ITS4 using conventional PCR. These primers amplified one single fragment with 223-bp from soil

samples of Bavarian fields 2 and 4, with the same expected size of *C. beticola* mycelium (Figs. 20 and 21).

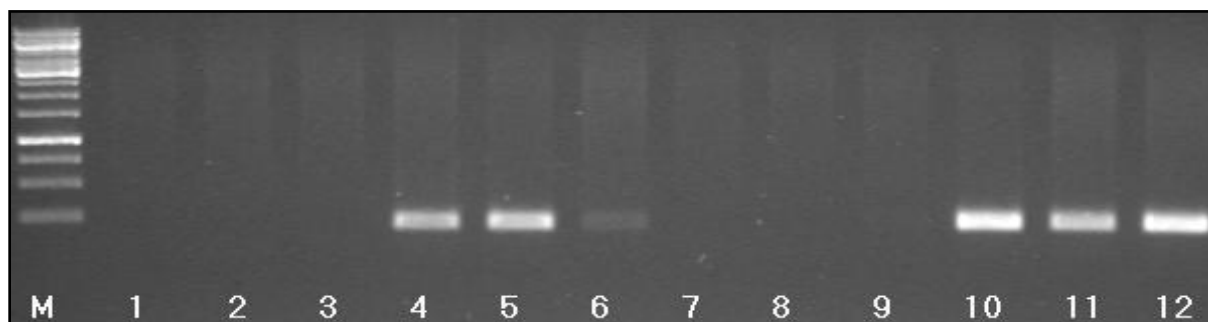


Fig. 20: PCR amplification of Bavarian fields soil DNA using the primer set ITS3/ ITS4. M is a 1000 bp marker, the lane from 1 to 12 are layer 1, 2 and 3 of field 1- layer 1, 2 and 3 of field 2- layer 1, 2 and 3 of field 3- layer 1, 2 and 3 of field 4.

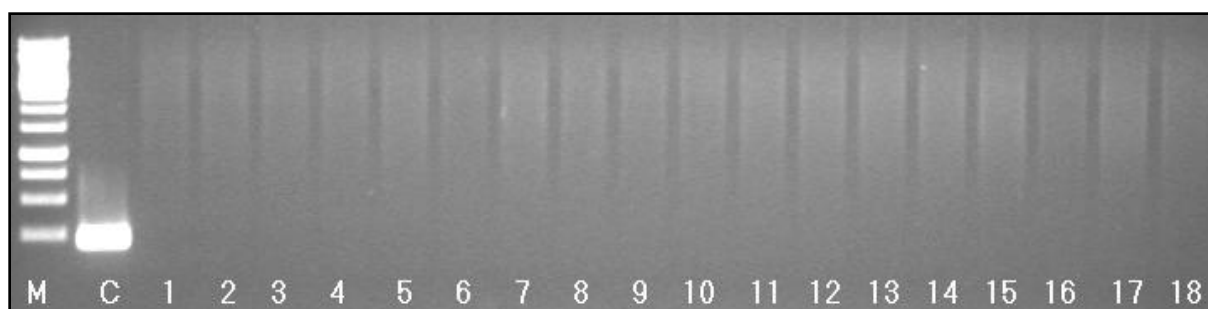


Fig. 21: PCR amplification of lower Saxonian fields soil DNA using the primer set ITS3/ ITS4. M is a 1000 bp marker, C is *C. beticola* mycelium (isolate Cb12), the lane from 1 to 18 are layer 1, 2 and 3 of field 1- layer 1, 2 and 3 of field 2- layer 1, 2 and 3 of field 3- layer 1, 2 and 3 of field 4- layer 1, 2 and 3 of field 5- layer 1, 2 and 3 of field 6.

Four amplified DNA fragments of the soil samples and one fragment of *C. beticola* mycelium (isolate Cb12) were then cut out and purified from the agarose gel using the QIAquick Gel Extraction Kit as described in material and methods. For analysis of the DNA sequence, the samples were sent to Eurofins MWG Operon. The comparison of both amplicon sequences mycelium (isolate Cb12) and field soil samples showed a high degree of accordance (Fig. 22), thus providing evidence that the fragments originate all from *C. beticola*.

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          *      20      *      40      *      60
4_ITS-3   : -----AACTTTACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGC : 55
MYCELLIUM_ : TTAACA AACTTTACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGC : 61
3_ITS-3   : -----AACTTTACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGC : 55
1_ITS-3   : -TAACA AACTTTACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGC : 60
2_ITS-3   : -----AACTTTACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGC : 55
          AACTTTACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGC

          *      80      *      100     *      120
4_ITS-3   : GATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCC : 116
MYCELLIUM_ : GATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCC : 122
3_ITS-3   : GATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCC : 116
1_ITS-3   : GATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCC : 121
2_ITS-3   : GATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCC : 116
          GATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCC

          *      140     *      160     *      180
4_ITS-3   : CCTTGGTATTCCGAGGGGCATGCCTGTTGAGCGTCATTTCAACCACTCAAGCCTCAAAACA : 177
MYCELLIUM_ : CCTTGGTATTCCGAGGGGCATGCCTGTTGAGCGTCATTTCAACCACTCAAGCCTCAAAACA : 183
3_ITS-3   : CCTTGGTATTCCGAGGGGCATGCCTGTTGAGCGTCATTTCAACCACTCAAGCCTCAGAACA : 177
1_ITS-3   : CCTTGGTATTCCGAGGGGCATGCCTGTTGAGCGTCATTTCAACCACTCAAGCCTCAAAACT : 182
2_ITS-3   : CCTTGGTATTCCGAGGGGCATGCCTGTTGAGCGTCATTTCAACCACTCAAGCCTCAAAACT : 177
          CCTTGGTATTCCGAGGGGCATGCCTGTTGAGCGTCATTTCAACCACTCAAGCCTCAaAAC

4_ITS-3   : G- : 178
MYCELLIUM_ : T- : 184
3_ITS-3   : A- : 178
1_ITS-3   : AG : 184
2_ITS-3   : G- : 178

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Fig. 22: DNA sequence of four soil samples of Bavarian fields 2 and 4 compared with *C. beticola* mycelium using the primer ITS3.

The real-time PCR method was applied for the quantification of soil DNA. For this purpose, a 10-fold dilution series from *C. beticola* pure mycelium was prepared; 10 ng, 1 ng, 0.1 ng, 0.01 ng and 0.001 ng. A good linear relationship was observed between the logs of *C. beticola* DNA and the values of cycle numbers obtained from real-time PCR (Fig. 23). Moreover, the amplification profile showed that a good reproducibility was obtained in 10 ng, 1 ng, 0.1 ng, 0.01 ng throughout 35 cycles (Fig. 24). To identify the target PCR products of the primer set ITS3 / ITS4, a dissociation curve was prepared using DNA of *C. beticola* mycelium and fields soil samples (Fig. 25). According to the dissociation curve, the melting temperature maximum of the target product, *C. beticola* DNA, was 80.1°C.

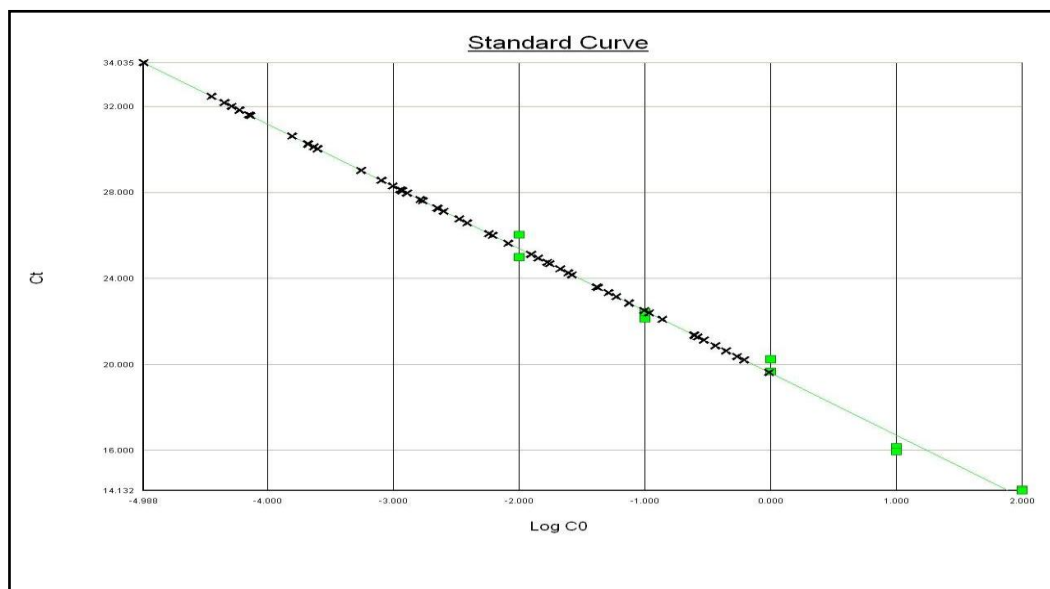


Fig. 23: Standard curve for the calculation of *Cercospora beticola* DNA amount based on the respective cycle number obtained from the real-time PCR.

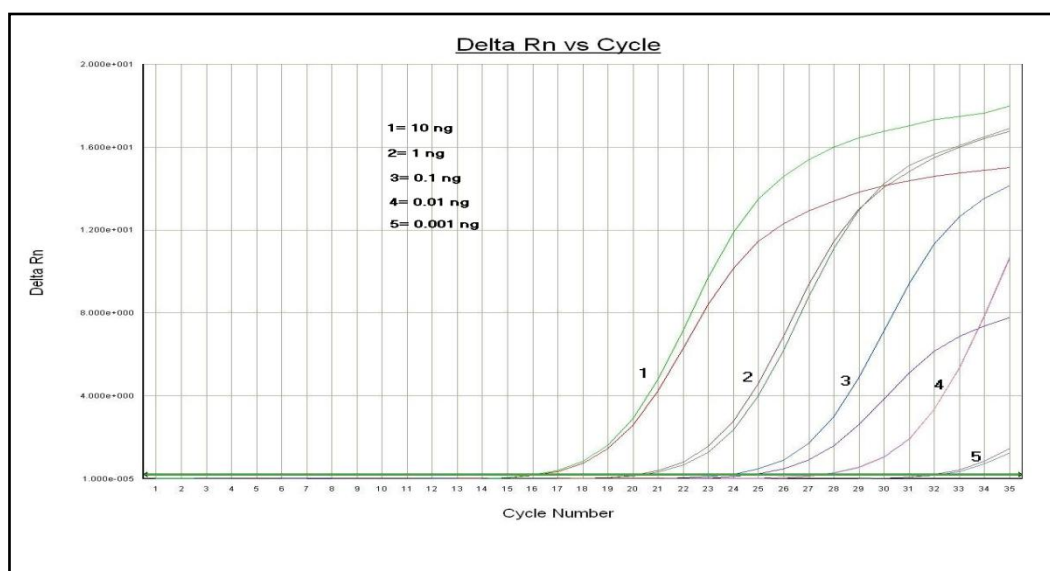


Fig. 24: The amplification curve of *Cercospora beticola* DNA dilutions obtained from the real-time PCR during 35 cycles.

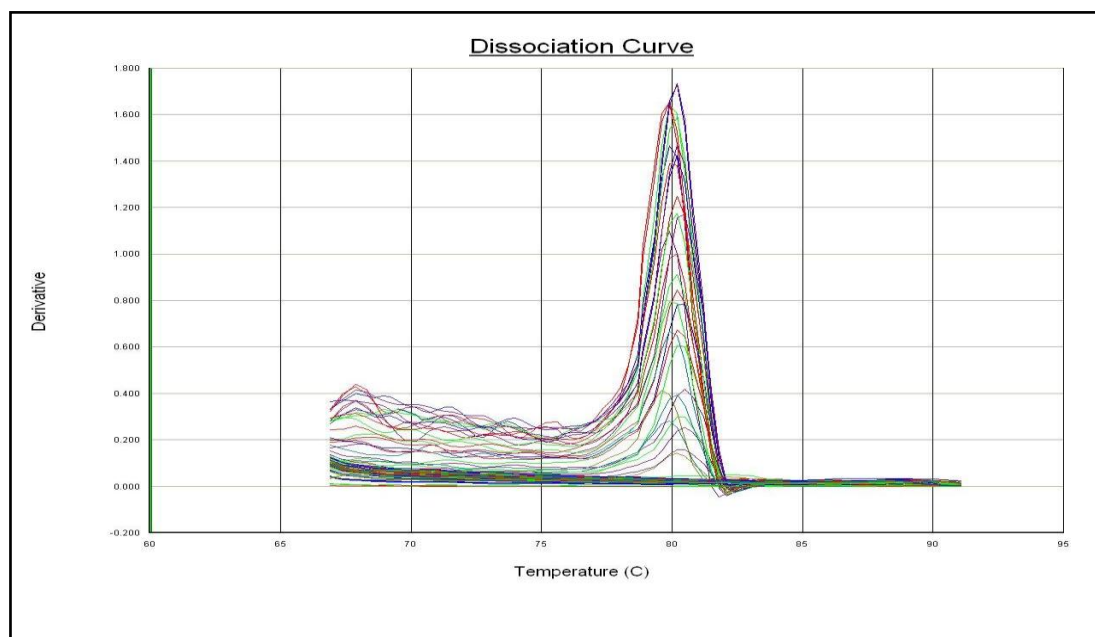


Fig. 25: The dissociation curve of the primer set ITS3 / ITS4 generated by real-time PCR using DNA of *Cercospora beticola* pure culture and fields soil samples.

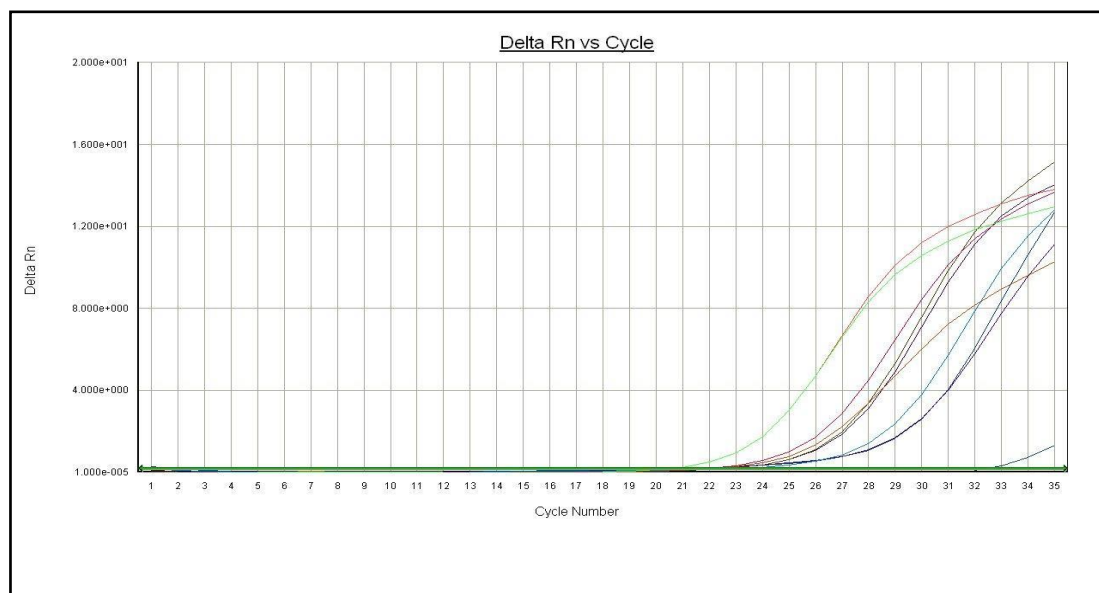


Fig. 26: The amplification curve of DNA Bavarian field soil samples obtained from the real-time PCR during 35 cycles.

3.6.1.1 Quantification of *C. beticola* DNA in Bavarian field 1

This field was cultivated with sugar beet 2 years before sampling date. The average DNA amounts of each layer in this field showed (Fig. 27 and Table A1) that the third layer contained the highest DNA-amount with 65.9 ng/g soil on average, while the first and second layers showed lower DNA amount with 9.283 and 0.881 ng/g soil, respectively. The DNA amount in the first layer ranged from 45.50 to 0 ng/g soil, while in the second and third layers it ranged from 6.27 to 0 and from 316.48 to 0 ng/g soil, respectively.

The differences between the layers in this field are not significant, where the probability of significant difference (P) between the first and each of second and third layer ($P = 0.83$ and 0.15 , respectively), while it was 0.10 between the second and third layer.

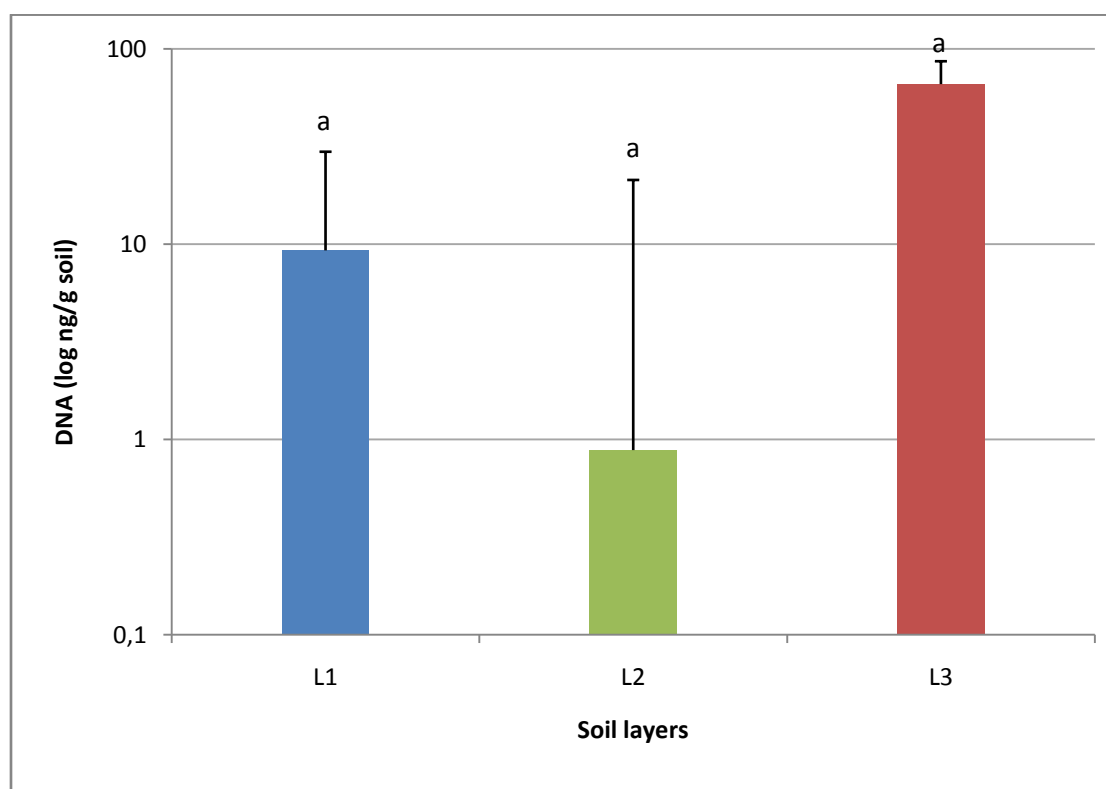


Fig. 27: The average DNA amounts (mean of $n=10$ soil samples) of Bavarian field 1 using real-time PCR. Error bars indicate standard error (Tukey test, $P<0.05$).

3.6.1.2 Quantification of *C. beticola* DNA in Bavarian field 2

This field was cultivated with sugar beet 1 year before sampling date. The average DNA amounts of each layer in this field showed (Fig. 28 and Table A2) that the first layer contained the highest DNA-amount with 120.895 ng/g soil on average, followed by the second and third layers with 43.809 and 23.202 ng/g soil, respectively. The DNA amount in the first layer ranged from 605.98 to 5.72 ng/g soil, while in the second and third layers ranged from 198.95 to 0.29 and from 169.41 to 0 ng/g soil, respectively.

The differences between the layers in this field are significant between the first and each of second and third layer ($P = 0.052$ and 0.014 , respectively), while it is not significant between the second and third layer ($P = 0.60$).

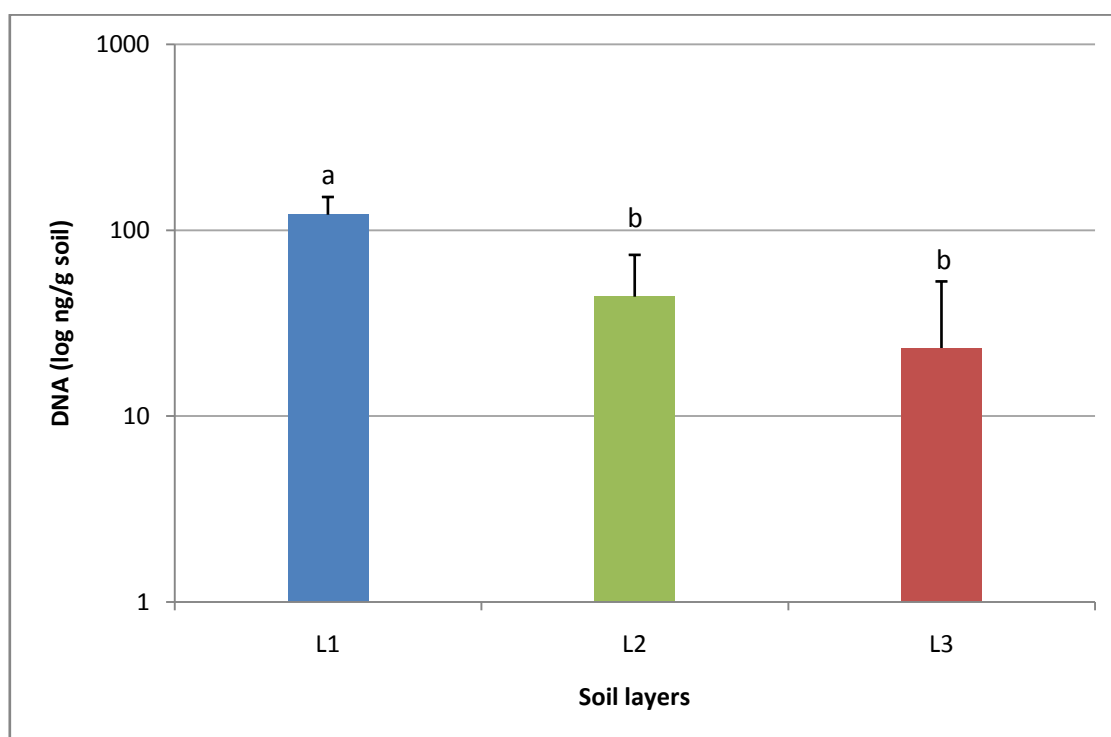


Fig. 28: The average DNA amounts (mean of $n=10$ soil samples) of Bavarian field 2 using real-time PCR. Error bars indicate standard error (Tukey test, $P<0.05$).

3.6.1.3 Quantification of *C. beticola* DNA in Bavarian field 3

This field was cultivated with sugar beet 3 years before sampling date. The average DNA amounts of each layer in this field showed (Fig. 29 and Table A3) that the first layer contained the highest DNA-amount with 5.687 ng/g soil on average, while the second and third layers showed lower DNA amount with 0.344 and 0.068 ng/g soil, respectively. The DNA amount in the first layer ranged from 20.61 to 0 ng/g soil, while in the second and third layers ranged from 1.02 to 0 and from 0.68 to 0 ng/g soil, respectively.

The differences between the layers in this field are not significant, where the probability of significant difference (P) between the first and each of second and third layer ($P = 0.89$ and 0.88 , respectively), while it was 0.99 between the second and third layer.

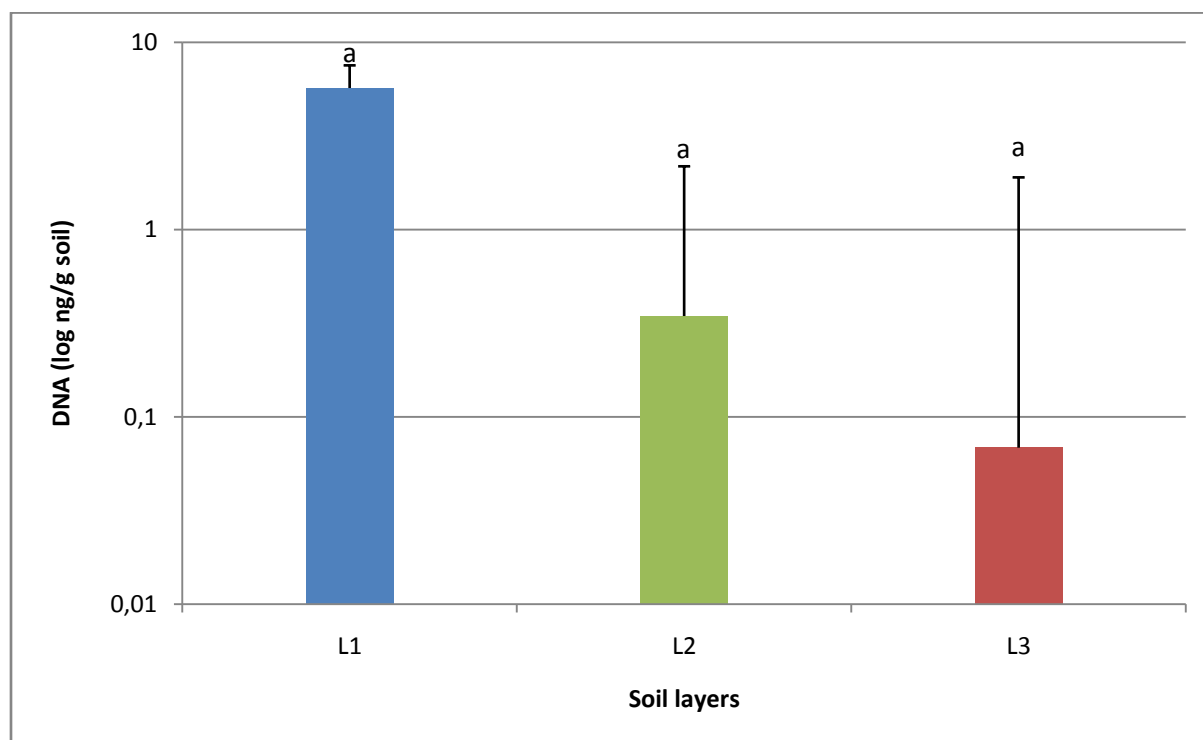


Fig. 29: The average DNA amounts (mean of $n=10$ soil samples) of Bavarian field 3 using real-time PCR. Error bars indicate standard error (Tukey test, $P<0.05$).

3.6.1.4 Quantification of *C. beticola* DNA in Bavarian field 4 and its degradation after 1 year

This field was cultivated with sugar beet 1 year before sampling date. Moreover, reduced tillage by cultivator let leaf debris mostly remain of soil surface. The average DNA amounts of each layer in this field showed (Fig. 30 and Table A4) that the second layer contained the highest DNA-amount with 204.729 ng/g soil on average, followed by the third layer which contained 144.129 ng/g soil, while the first layer showed lower DNA amount with 19.616 ng/g soil. The DNA amount in the first layer ranged from 107.34 to 1.82 ng/g soil, while in the second and third layers ranged from 752.51 to 0.79 and from 526.71 to 7.88 ng/g soil, respectively.

The differences between the layers in this field are significant between the first and each of second and third layer ($P < 0.0001$ and $= 0.001$, respectively), while it is not significant between the second and third layer ($P = 0.12$).

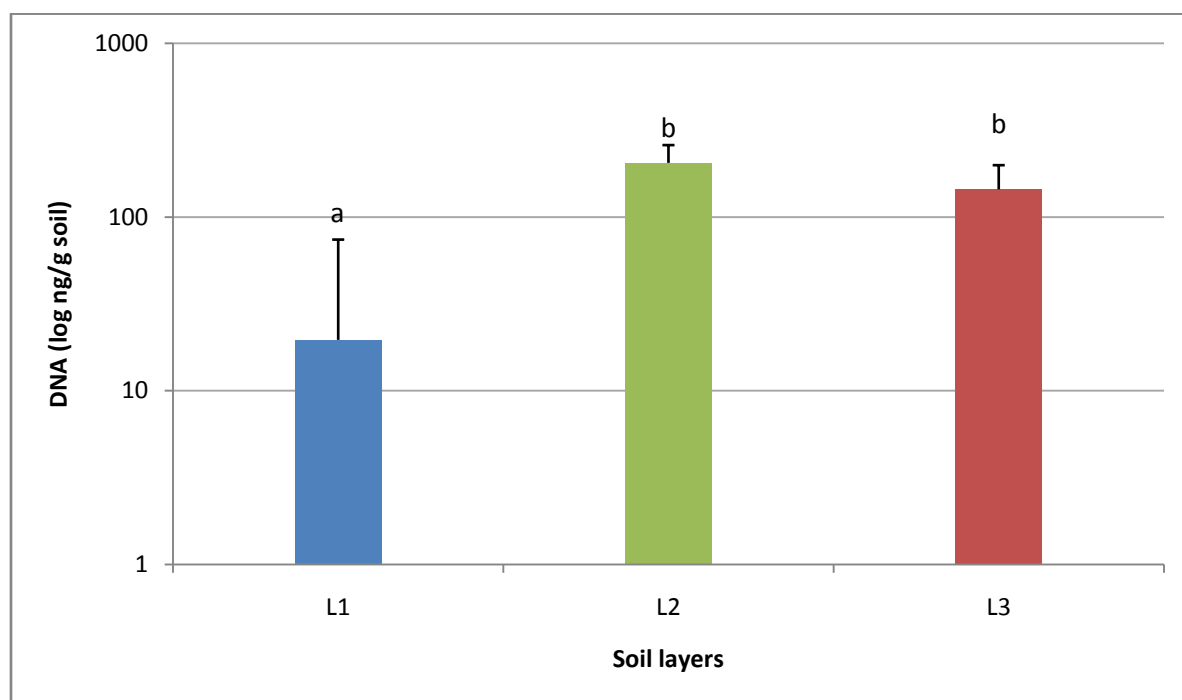


Fig. 30: The average DNA amounts (mean of n=10 soil samples) of Bavarian field 4 using real-time PCR. Error bars indicate standard error (Tukey test, $P < 0.05$).

Four soil samples from each layer were taken from field 4, 1 year after the first sampling as described in materials and methods to determine the degradation of *C. beticola* inoculum.

Compared with the results of field 4 in 2010, the average DNA amounts declined after 1 year in the second layer from 204.72 to 8.47 ng/g soil and in the third layer from 144.12 to 0.01 ng/g soil. However, it increased in the first layer from 19.61 to 73.77 ng/g soil. The average DNA amounts of each layer in this field showed (Fig. 31 and Table A5) that the first layer contained the highest DNA-amount with 73.77 ng/g soil on average, followed by the second layer which contained 8.47 ng/g soil, while the third layer showed lower DNA amount with 0.010ng/g soil.

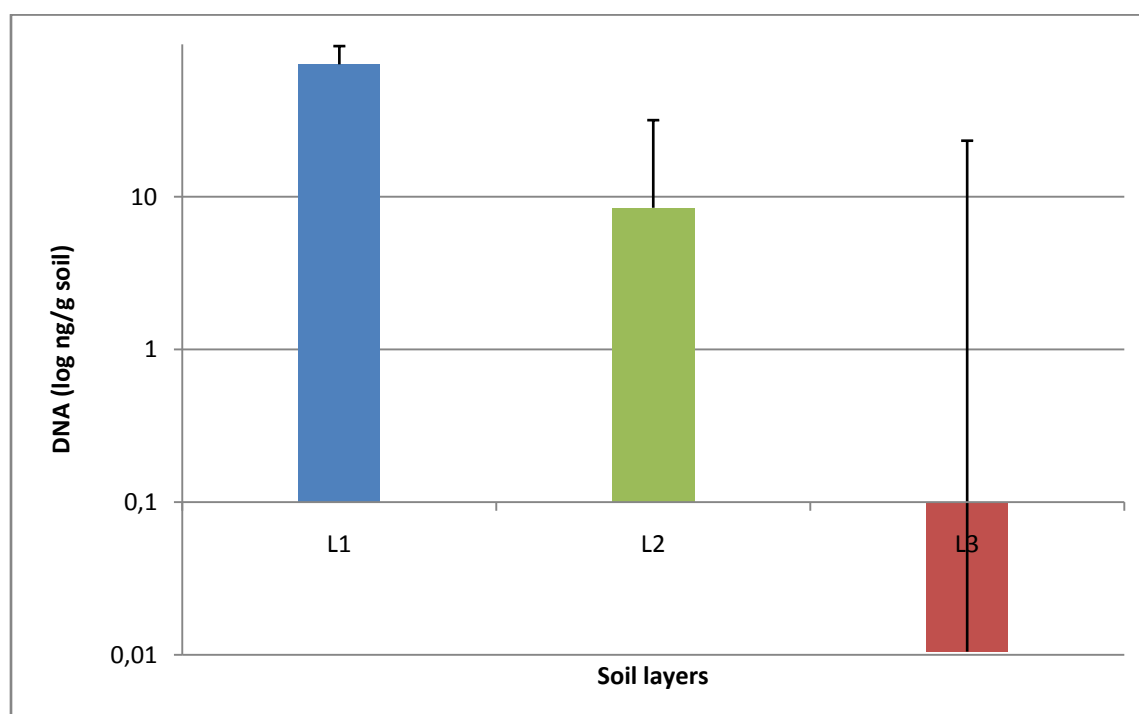


Fig. 31: The average DNA amounts (mean of $n = 4$ soil samples) of Bavarian field 4, 1 year after the first sampling date, using real-time PCR. Error bars indicate standard error.

3.6.1.5 Comparison between the four Bavarian fields

The comparison showed that the field containing the highest amount of inoculum DNA was field 4 with a total amount of DNA with 3684.74 ng/ g soil (which had been cultivated with sugar beet 1 year before sampling date), followed by field 2 with a total amount of DNA with 1879.06 ng/g soil (which had been cultivated with sugar beet 1 year before sampling date). Field 1 came in the third order with a total amount of DNA with 760.65 ng/g soil (which had been cultivated with sugar beet 2 years before sampling date), while field 3 contained the lowest amounts of inoculum DNA with a total amount of DNA with 61.007 ng/g soil (which had been cultivated 3 years before sampling date) (Fig. 32). The differences are significant between field 1 and field 4 ($P < 0.0001$), field 2 and field 3 ($P = 0.0084$), field 2 and field 4 ($P = 0.0088$), field 3 and field 4 ($P < 0.0001$). The differences are not significant between field 1 and field 2 ($P = 0.10$), field 1 and field 3 ($P = 0.30$).

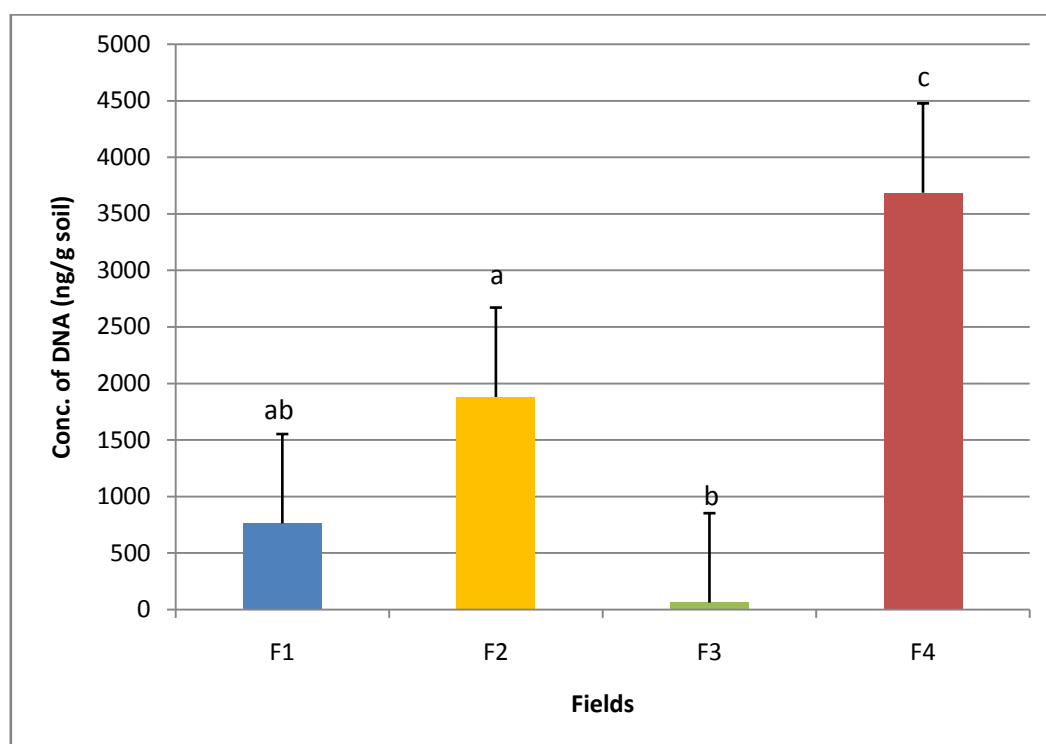


Fig. 32: Comparison of soil DNA amounts between the four Bavarian fields. Error bars indicate standard error (Tukey test, $P < 0.05$).

3.6.1.6 Quantification of *C. beticola* DNA in Lower Saxonian field 1

This field was cultivated with sugar beet 3 years before sampling date. The average DNA amounts in this field showed (Fig. 33 and Table A6) that the second layer contained the highest DNA-amount with 1.916 ng/g soil on average, while the first and third layers showed lower DNA amount with 1.659 and 0.495 ng/g soil, respectively. The DNA amount in the first layer ranged from 9.01 to 0 ng/g soil, while in the second and third layers it ranged from 14.93 to 0 and from 3.21 to 0 ng/g soil, respectively.

The differences between the layers in this field are not significant, where the probability of significant difference (P) between the first and each of second and third layer ($P = 0.99$ and 0.97 , respectively), while it was 0.97 between the second and third layer.

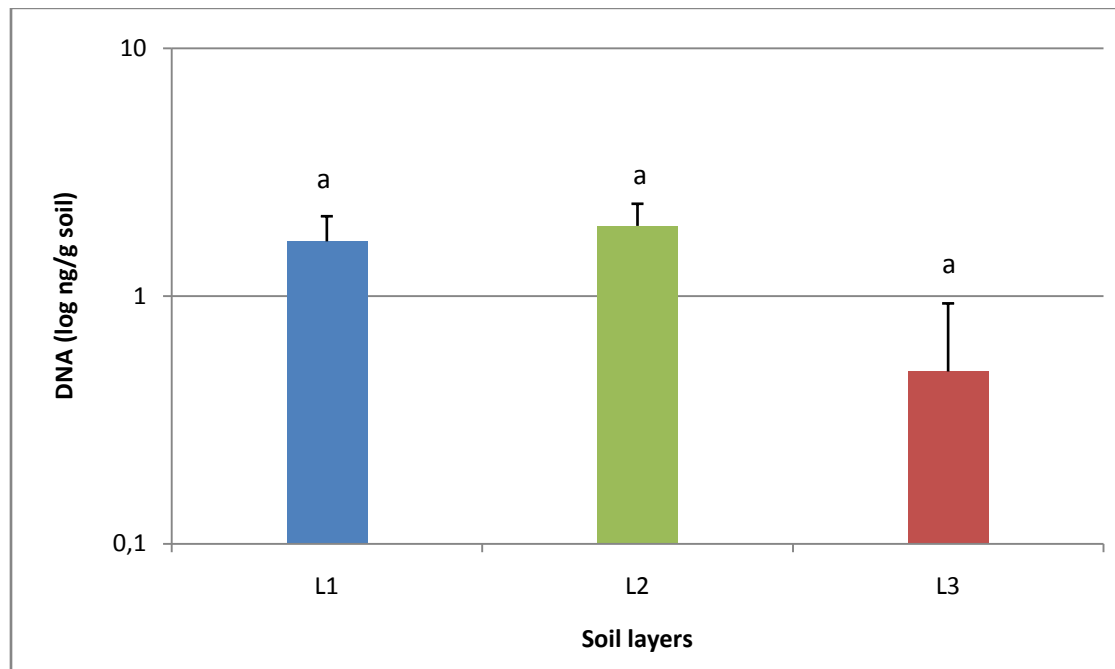


Fig. 33: The average DNA amounts (mean of $n=10$ soil samples) of Lower Saxonian field 1 using real-time PCR. Error bars indicate standard error (Tukey test, $P<0.05$).

3.6.1.7 Quantification of *C. beticola* DNA in Lower Saxonian field 2

This field was cultivated with sugar beet 2 years before sampling date. The average DNA amounts of each layer in this field showed (Fig. 34 and Table A7) that the first layer contained the highest DNA-amount with 0.314 ng/g soil on average, followed by the third and second layers with 0.182 and 0.11 ng/g soil, respectively.

The differences between the layers in this field are not significant, where the probability of significant difference (P) between the first and each of second and third layer ($P = 0.99$ and 0.99 , respectively), while it was 0.99 between the second and third layer.

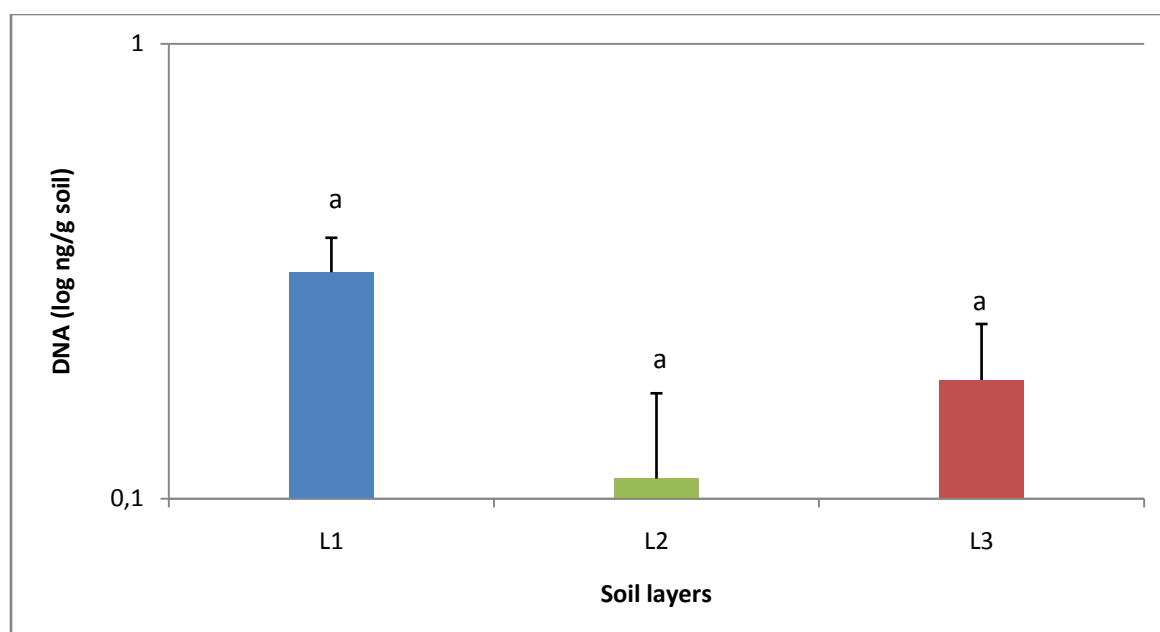


Fig. 34: The average DNA amounts (mean of $n=10$ soil samples) of Lower Saxonian field 2 using real-time PCR. Error bars indicate standard error (Tukey test, $P<0.05$).

3.6.1.8 Quantification of *C. beticola* DNA in Lower Saxonian field 3

This field was cultivated with sugar beet 1 year before sampling date. The average DNA amounts of each layer in this field showed (Fig. 35 and Table A8) that the second layer contained the highest DNA-amount with 80.171 ng/g soil on average, followed by the third and first layers with 14.825 and 2.970 ng/g soil, respectively. The DNA amount in the first layer ranged from 26.13 to 0 ng/g soil, while in the second and third layers it ranged from 503.28 to 0 and from 107.84 to 0 ng/g soil, respectively.

The differences between the layers in this field are not significant, where the probability of significant difference (P) between the first and each of second and third layer ($P = 0.05$ and 0.76 , respectively), while it was 0.09 between the second and third layer.

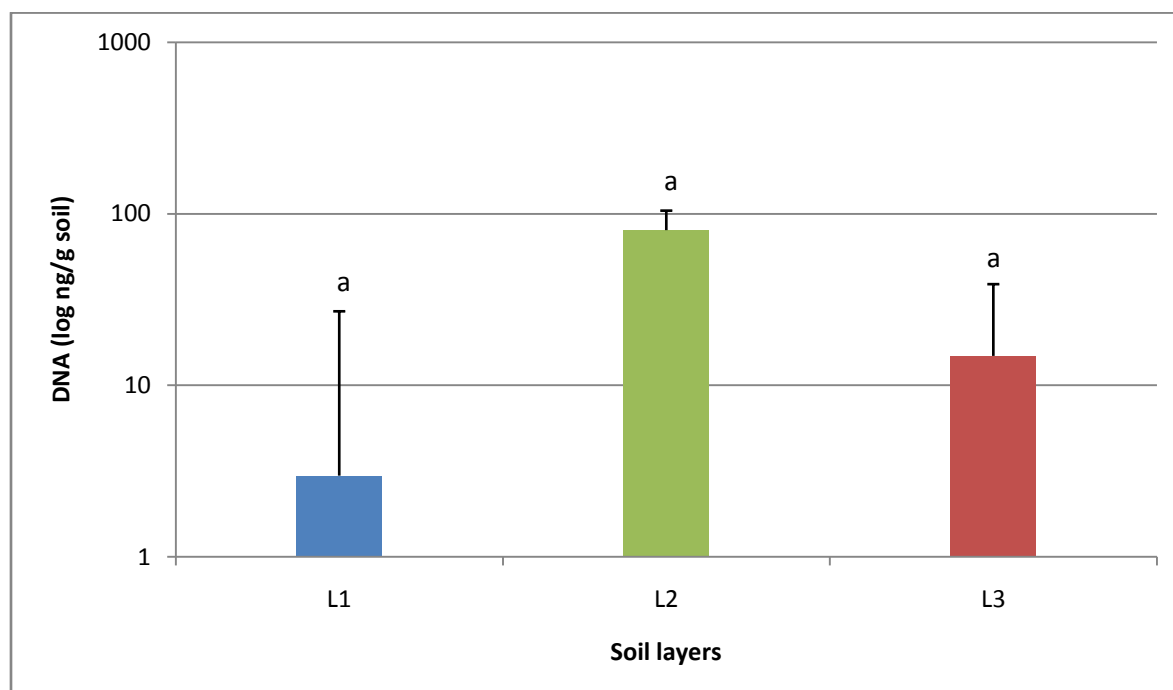


Fig. 35: The average DNA amounts (mean of $n=10$ soil samples) of Lower Saxonian field 3 using real-time PCR. Error bars indicate standard error (Tukey test, $P<0.05$).

3.6.1.9 Quantification of *C. beticola* DNA in Lower Saxonian field 4

This field was cultivated with sugar beet 3 years before sampling date. The average DNA amounts of each layer in this field showed (Fig. 36 and Table A9) that the third layer contained the highest DNA-amount with 0.872 ng/g soil on average, followed by the second and first layers with 0.612 and 0.454 ng/g soil, respectively.

The differences between the layers in this field are not significant, where the probability of significant difference (P) between the first and each of second and third layer ($P = 0.99$ and 0.99 , respectively), while it was 0.99 between the second and third layer.

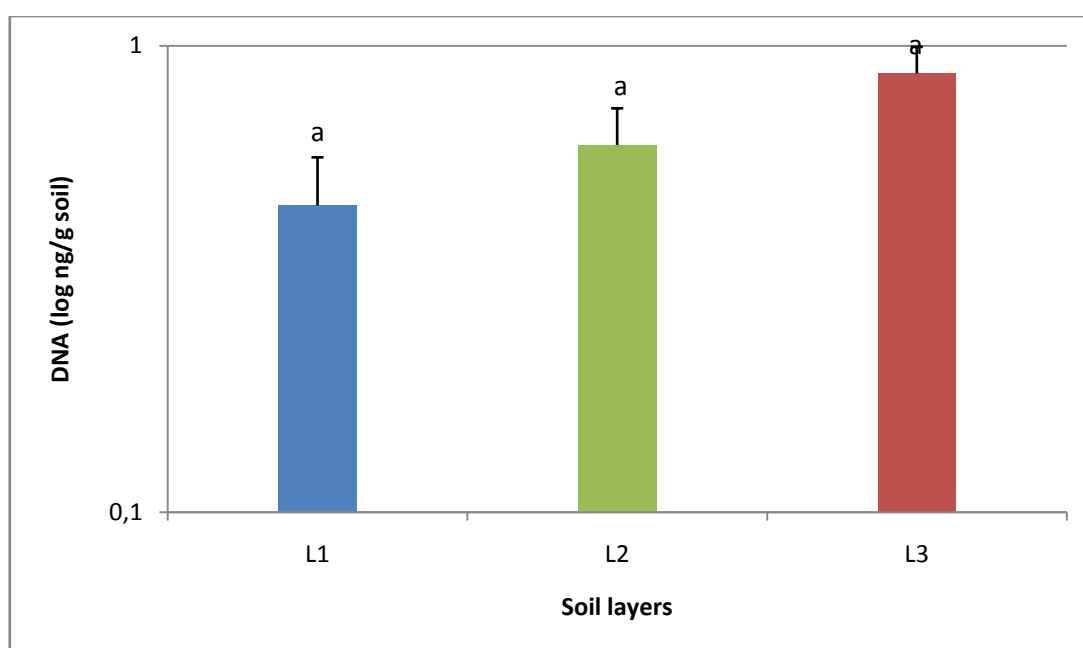


Fig. 36: The average DNA amounts (mean of $n=10$ soil samples) of Lower Saxonian field 4 using real-time PCR. Data are means of ten samples. Error bars indicate standard error (Tukey test, $P<0.05$).

3.6.1.10 Quantification of *C. beticola* DNA in Lower Saxonian field 5

This field was cultivated with sugar beet 1 year before sampling date. The average DNA amounts of each layer in this field showed (Fig. 37 and Table A10) that the third layer contained the highest DNA-amount with 60.924 ng/g soil on average, followed by the first and second layers with 54.702 and 2.332 ng/g soil, respectively. The DNA amount in the first layer ranged from 262.79 to 0 ng/g soil, while in the second and third layers it ranged from 22.01 to 0 and from 606.10 to 0 ng/g soil, respectively.

The differences between the layers in this field are not significant, where the probability of significant difference (P) between the first and each of second and third layer ($P = 0.18$ and 0.87 , respectively), while it was 0.13 between the second and third layer.

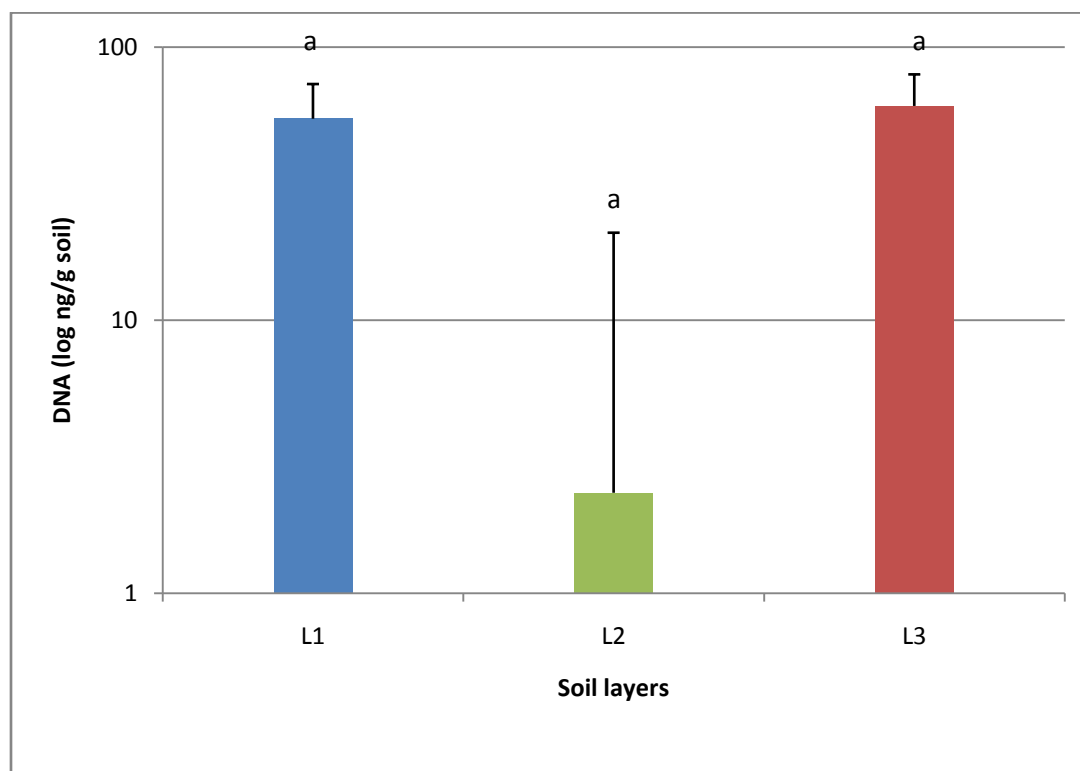


Fig. 37: The average DNA amounts (mean of $n=10$ soil samples) of Lower Saxonian field 5 using real-time PCR. Error bars indicate standard error (Tukey test, $P<0.05$).

3.6.1.11 Quantification of *C. beticola* DNA in Lower Saxonian field 6

This field was cultivated with sugar beet 3 years before sampling date. The average DNA amounts of each layer in this field showed (Fig. 38 and Table A11) that the second layer contained the highest DNA-amount with 1.972 ng/g soil on average, followed by the first and third layers with 1.957 and 0.965 ng/g soil, respectively.

The differences between the layers in this field are not significant, where the probability of significant difference (P) between the first and each of second and third layer ($P = 0.99$ and 0.98 , respectively), while it was 0.97 between the second and third layer.

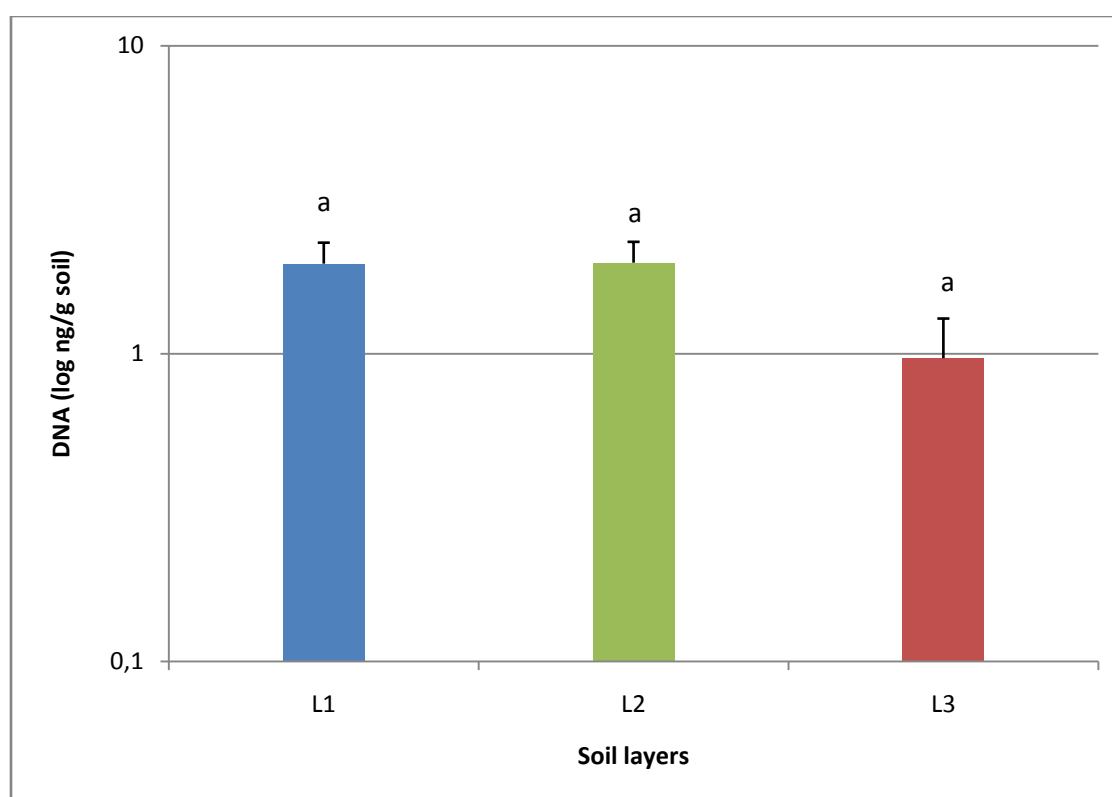


Fig. 38: The average DNA amounts (mean of $n=10$ soil samples) of Lower Saxonian field 6 using real-time PCR. Error bars indicate standard error (Tukey test, $P<0.05$).

3.6.1.12 Comparison between the six Lower Saxonian fields

The comparison showed that the field containing the highest amount of inoculum DNA was field 5 with 1179.59 ng/g soil (which had been cultivated with sugar beet 1 year before sampling date), followed by field 3 with a total amount of 979.67 ng/g soil (which had been cultivated with sugar beet 1 year before sampling date). Field 6 came in the third order, where it contained a total amount of DNA with 48.95 ng/g soil (which had been cultivated with sugar beet 3 years before sampling date), followed by field 1 with a total amount of DNA with 40.71 ng/g soil (which had been cultivated with sugar beet 3 years before sampling date). Field 4 came in the fifth order, where it contained a total amount of DNA with 19.38 ng/g soil (which had been cultivated with sugar beet 3 years before sampling date), while the field contained the lowest inoculum DNA was field 2 with a total amount of DNA with 6.07 ng/g soil (which had been cultivated 2 years before sampling date) (Fig. 39). The differences are not significant between all Lower Saxonian fields and each other.

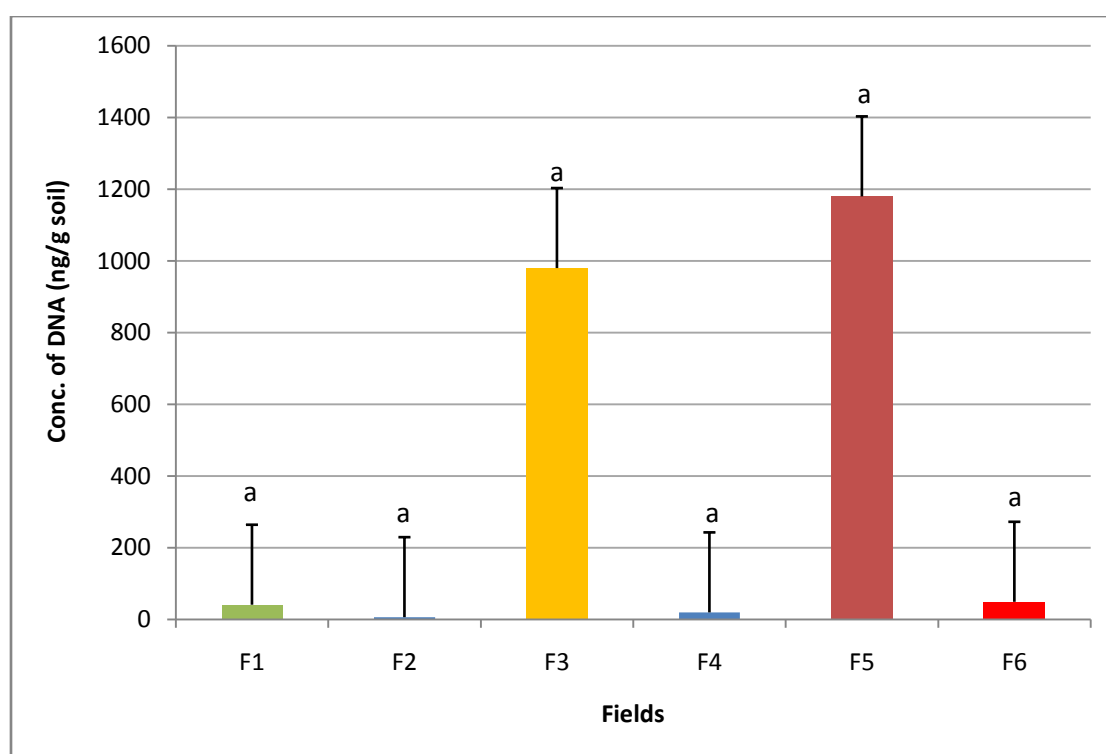


Fig. 39: Comparison of soil DNA amounts between the six Lower Saxonian fields. Error bars indicate standard error (Tukey test, $P < 0.05$).

3.6.1.13 Quantification of *C. beticola* DNA in USA field 1

This field was cultivated with sugar beet 2 years before sampling date and it maintained with conventional tillage after sugar beet cultivation. One sample was collected from each layer in this field. The results (Fig. 40 and Table A12) show that the first layer contained the highest DNA inoculum with amount of 87.35 ng/g soil, followed by the second layer which contained 11.10 ng/g soil, while the third layer did not contain detectable levels of DNA.

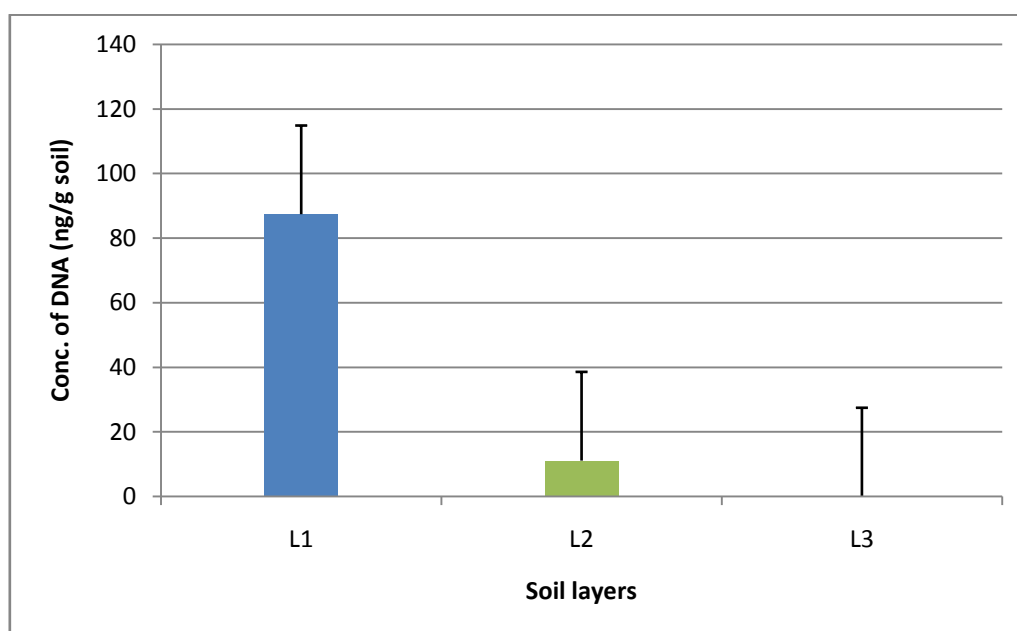


Fig. 40: Quantification of DNA amounts of USA field 1 using real-time PCR. Error bars indicate standard error.

3.6.1.14 Quantification of *C. beticola* DNA in USA field 2

This field was cultivated with sugar beet 2 years before sampling date and it maintained with strip tillage after sugar beet cultivation. One sample was collected from each layer in this field. The results (Fig. 41 and Table A13) show that the first layer contained the highest DNA inoculum with amount of 0.67 ng/g soil, followed by the second layer which contained 0.19 ng/g soil, while the third layer did not contain DNA.

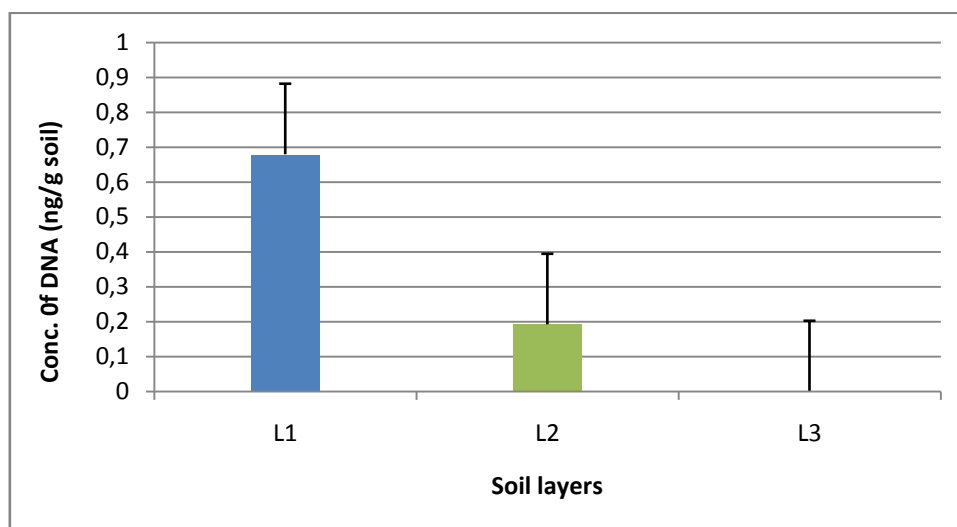


Fig. 41: Quantification of DNA amounts of USA field 2 using real-time PCR. Error bars indicate standard error.

3.6.1.15 Comparison between the two USA fields

The amount of DNA of the two USA fields samples were compared with each other. The results showed that field 1, which contained a total amount of DNA of 98.48 ng/g soil, was higher than field 2, which contained a total amount of DNA of 0.87 ng/g soil (Fig. 42).

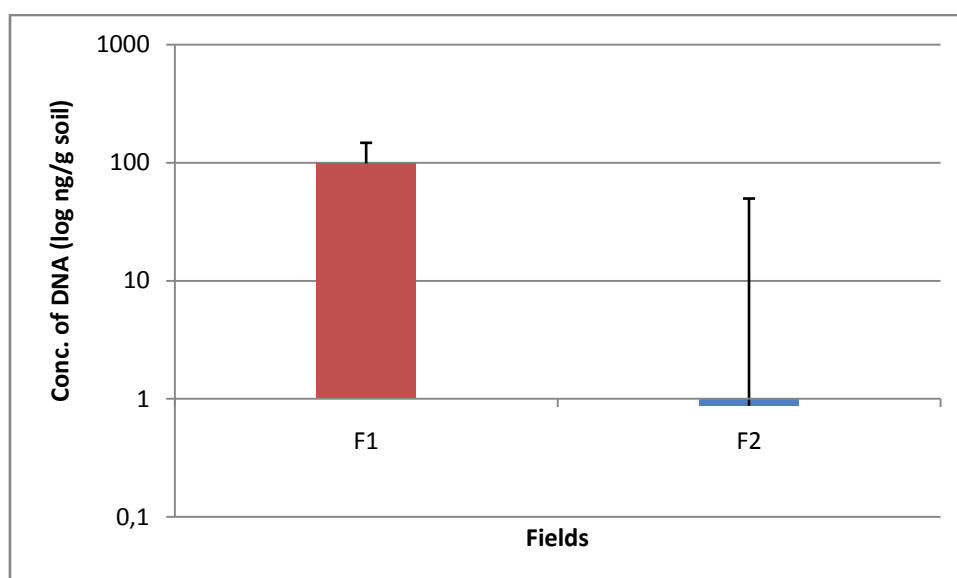


Fig. 42: Comparison of soil DNA amounts between the two USA fields. Error bars indicate standard error.

3.6.2 Quantification of *C. beticola* inoculum in fields soil using ELISA

The OD values in this experiment were correlated with amount of *C. beticola* mycelium as described in section 3.4.2 (determination of *C. beticola* inoculum in climate chamber using ELISA).

3.6.2.1 Quantification of *C. beticola* antigen in Bavarian field 1

This field was cultivated with sugar beet 2 years before sampling date. The average antigen amounts of each layer in this field showed (Fig. 43 and Table A15) that the third layer contained the highest-antigen amount with 199.333 μg mycelium/g soil on average, followed by the second and first layers with 184.333 and 151 μg mycelium/g soil, respectively. The antigen amount in the first layer in all samples was about 151 μg mycelium/g soil, while in the second layer it was 184.33 μg mycelium/g soil. The third layer contained 184.33 μg mycelium/g soil only in the first sample, while the other samples in the same layer contained 201 μg mycelium/g soil.

The differences between the layers in this field are significant between the first and each of second and third layer ($P < 0.0001$), while it was ($P = 0.028$) between the second and third layer.

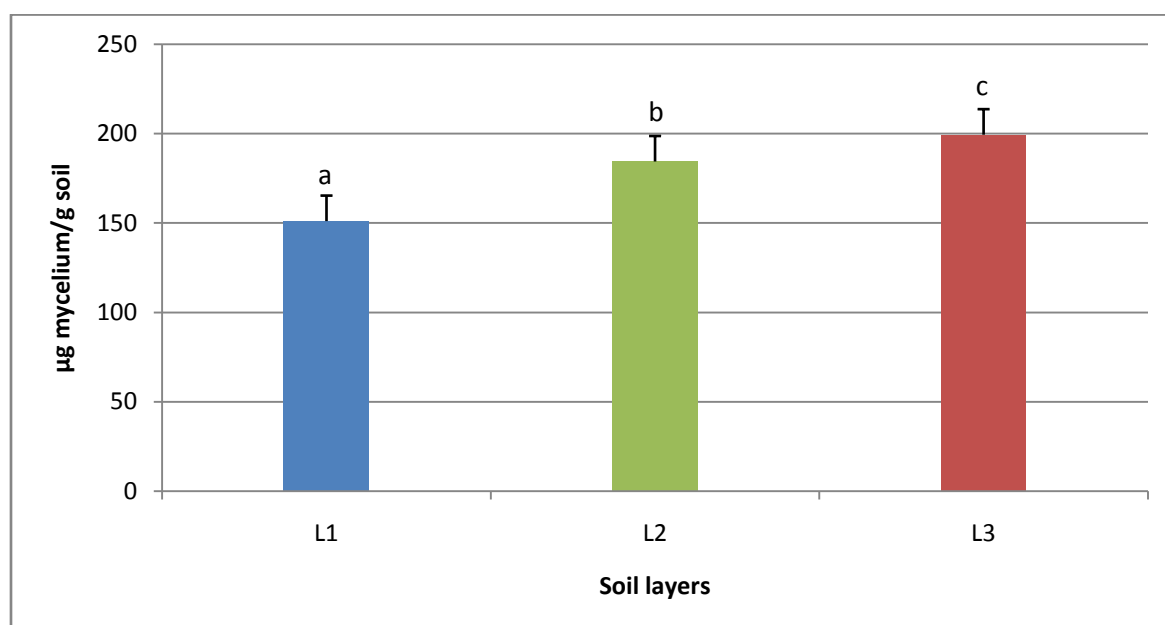


Fig. 43: The average antigen amounts (mean of $n=10$ soil samples) of Bavarian field 1 using ELISA. Error bars indicate standard error (Tukey test, $P < 0.05$).

3.6.2.2 Quantification of *C. beticola* antigen in Bavarian field 2

This field was cultivated with sugar beet 1 year before sampling date. The average antigen amounts of each layer in this field showed (Fig. 44 and Table A16) that the first layer contained the highest-antigen amount with 284.333 μg mycelium/g soil on average, followed by the third and second layers with 267.666 and 217.666 μg mycelium/g soil, respectively. The antigen amount in the first layer in all samples was about 284.33 μg mycelium/g soil, while in the second and third layers were 217.66 and 267.66 μg mycelium/g soil, respectively.

The differences between the layers in this field are significant between the first and each of second and third layer ($P < 0.0001$ and $= 0.014$, respectively), while it was ($P < 0.0001$) between the second and third layer.

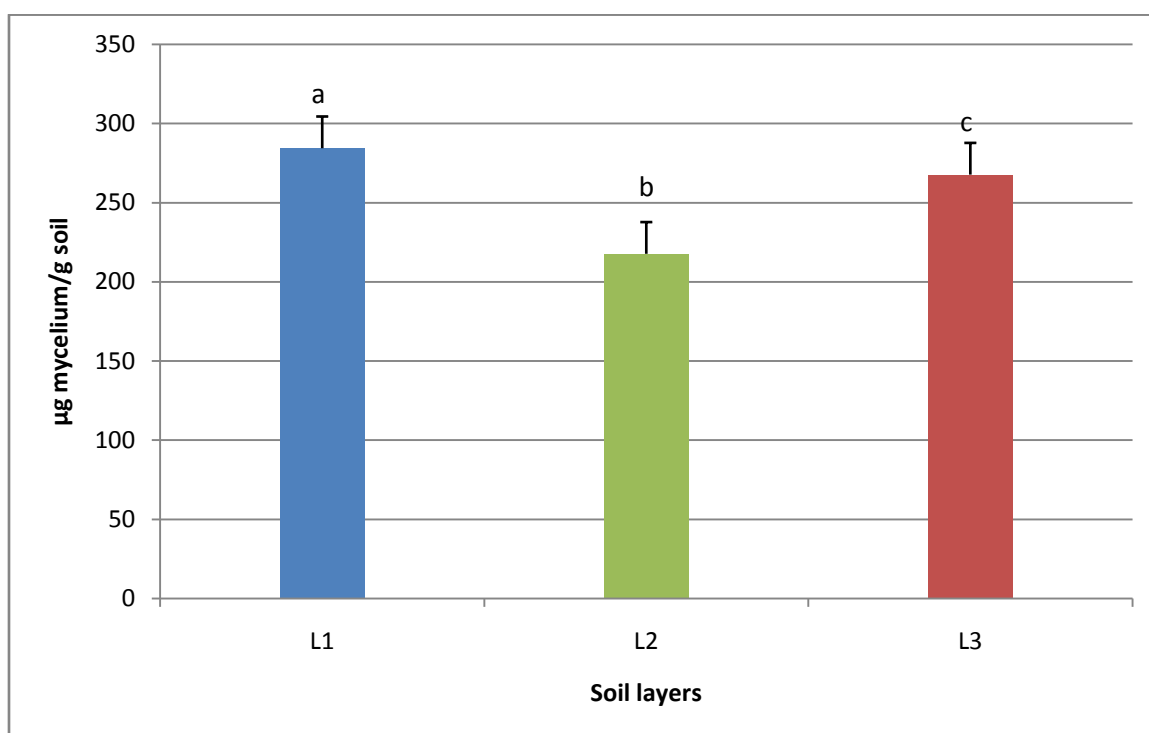


Fig. 44: The average antigen amounts (mean of $n=10$ soil samples) of Bavarian field 2 using ELISA. Error bars indicate standard error (Tukey test, $P<0.05$).

3.6.2.3 Quantification of *C. beticola* antigen in Bavarian field 3

This field was cultivated with sugar beet 3 years before sampling date. The average antigen amounts of each layer in this field showed (Fig. 45 and Table A17) that the second layer contained the highest-antigen amount with 167.666 μg mycelium/g soil on average, followed by the first and third layers with 164.333 and 154.333 μg mycelium/g soil, respectively.

The antigen amount in the first layer of samples 3 and 6 was lower than in the other samples, with about 151 μg mycelium/g soil, while in the same layer of the other samples were 167.66 μg mycelium/g soil. In contrast, the third layer of sample 2 and 4 were higher than in the other samples with about 167.66 μg mycelium/g soil, while the other samples were 151 μg mycelium/g soil. The second layer contained the same antigen amount in all samples with about 167.66 μg mycelium/g soil.

The differences between the layers in this field are not significant, where the probability of significant difference (P) between the first and each of second and third layer ($P = 0.62$ and 0.14 , respectively), while it was 0.05 between the second and third layer.

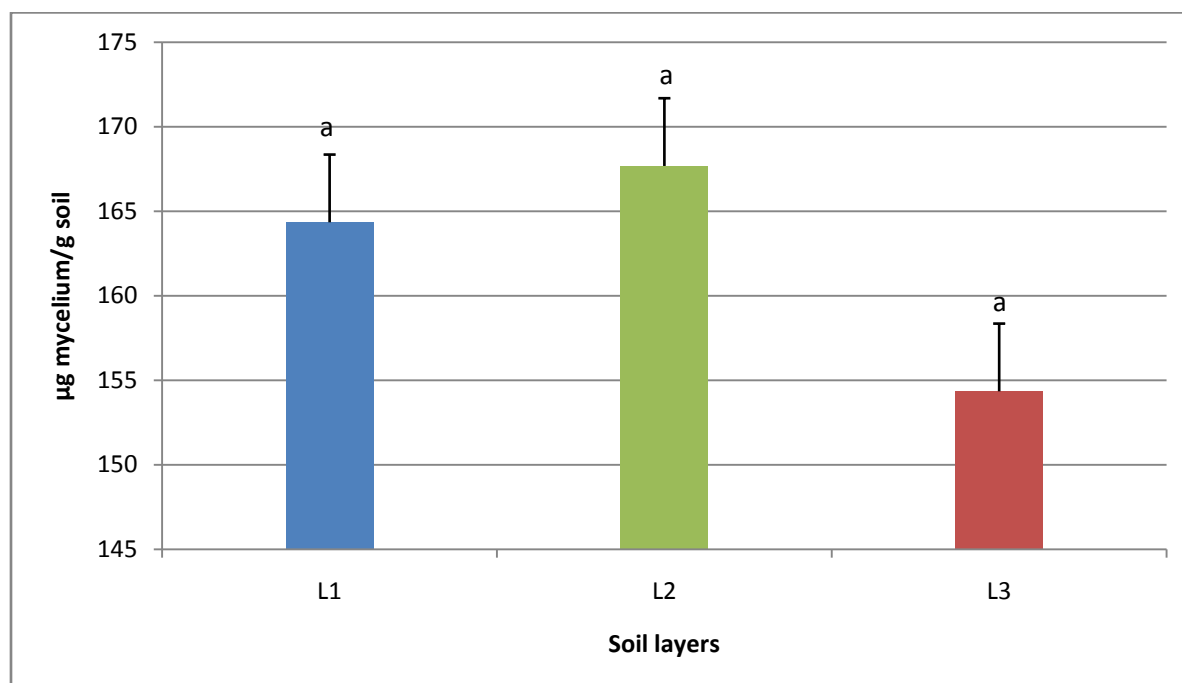


Fig. 45: The average antigen amounts (mean of $n=10$ soil samples) of Bavarian field 3 using ELISA. Error bars indicate standard error (Tukey test, $P<0.05$).

3.6.2.4 Quantification of *C. beticola* antigen in Bavarian field 4 and its degradation after 1 year

This field was cultivated with sugar beet 1 year before sampling date. The average antigen amounts of each layer in this field showed (Fig. 46 and Table A18) that the third layer contained the highest-antigen amount with 314.333 μg mycelium/g soil on average, followed by the second and first layers with 284.333 and 252.666 μg mycelium/g soil, respectively. The antigen amount in the first layer ranged from 251 to 267.66 μg mycelium/g soil, while in the second layer it ranged from 267.66 to 334.33 μg mycelium/g soil and in the third layer from 251 to 417.66 μg mycelium/g soil.

The differences between all layers in this field are significant ($P < 0.0001$).

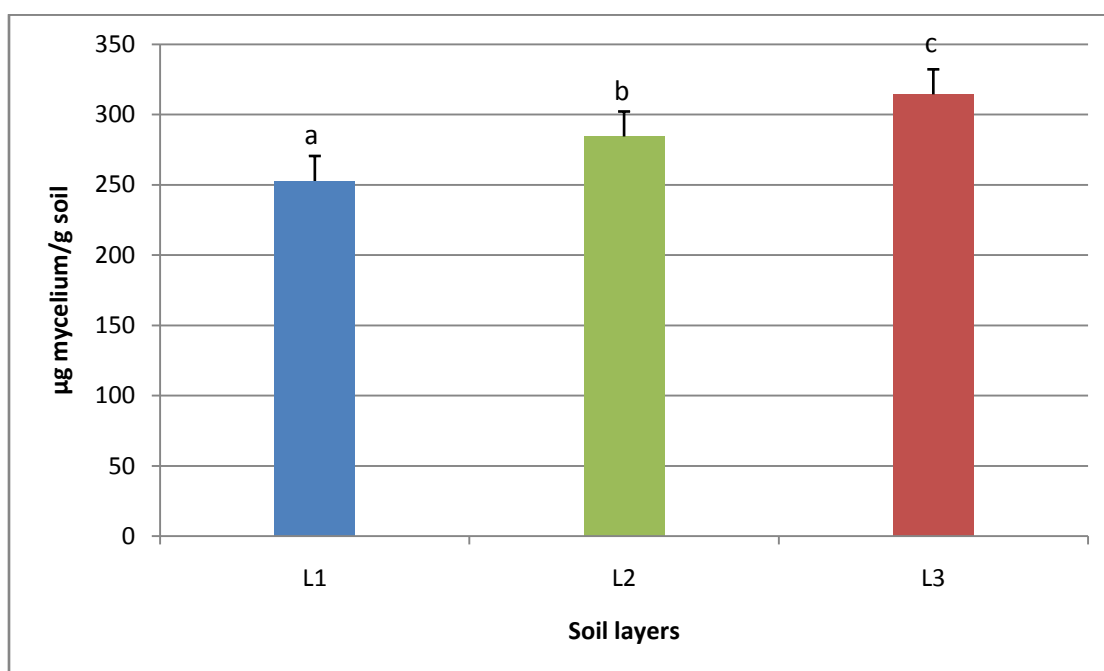


Fig. 46: The average antigen amounts (mean of $n=10$ soil samples) of Bavarian field 4 using ELISA. Error bars indicate standard error (Tukey test, $P < 0.05$).

Four soil samples from each layer were taken from field 4, 1 year after the first sampling as described in materials and methods to determine the degradation of *C. beticola* inoculum.

Compared with the results of field 4 in 2010, the antigen amounts were declined after 1 year. The average antigen amounts declined in the first layer from 252.66 to 201 μg mycelium/g soil and in the second layer from 284.33 to 167.66 μg mycelium/g soil, while it declined in the third layer from 314 to 151 μg mycelium/g soil. The average antigen amounts of samples of each layer in this field showed (Fig. 47 and Table A19) that the first layer contained the highest-antigen amount with 201 μg mycelium/g soil on average, followed by the second layer which contained 167.66 μg mycelium/g soil, while the third layer showed lower antigen amount with 151 μg mycelium/g soil.

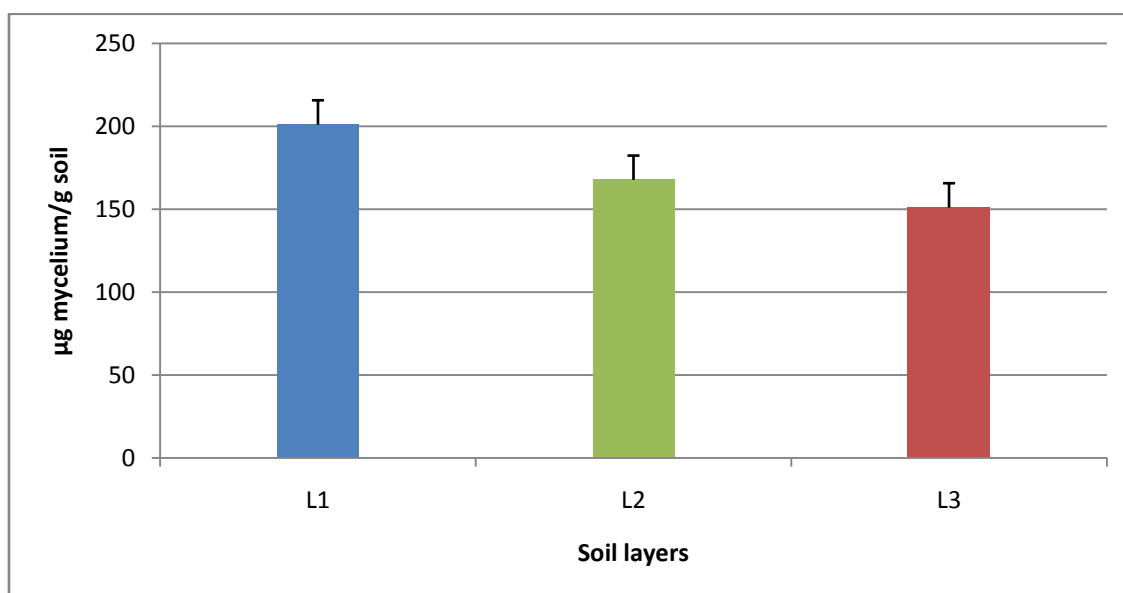


Fig. 47: The average antigen amounts (mean of $n = 4$ soil samples) of Bavarian field 4, 1 year after the first sampling date, using ELISA. Error bars indicate standard error.

3.6.2.5 Comparison between the four Bavarian fields

The comparison showed that the field contained the highest inoculum antigen was field 4 with a total amount of 8513.33 μg mycelium/g soil (which had been cultivated with sugar beet 1 year before sampling date), followed by field 2 with a total amount of antigen with 7696.66 μg mycelium/g soil (which had been cultivated with sugar beet 1 year before sampling date). Field 1 came in the third order, where it contained a total amount of antigen with 5346.66 μg mycelium/g soil (which had been cultivated with sugar beet 2 years before sampling date). The field contained the lowest inoculum antigen was field 3 with a total amount of 4863.33 μg mycelium/g soil (which had been cultivated 3 years before sampling date) (Fig. 48).

The differences between all Bavarian fields are significant ($P < 0.0001$).

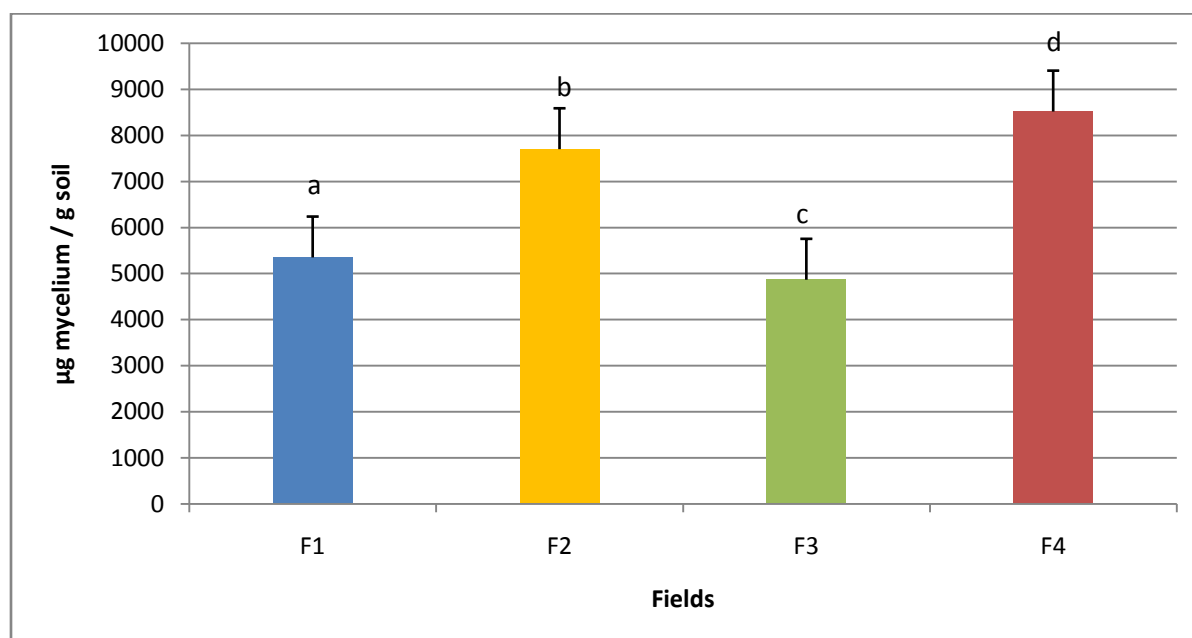


Fig. 48: Comparison of antigen amounts between the fourth Bavarian fields. Error bars indicate standard error (Tukey test, $P < 0.05$).

3.6.2.6 Quantification of *C. beticola* antigen in Lower Saxonian field 1

This field was cultivated with sugar beet 3 years before sampling date. The average antigen amounts of each layer in this field showed (Fig. 49 and Table A20) that the third layer contained the highest-antigen amount with 367.666 μg mycelium/g soil on average, followed by the first and second layers with 337.666 and 322.666 μg mycelium/g soil, respectively. The antigen amount in the first layer ranged from 317.66 to 384.33 μg mycelium/g soil, while in the second layer it ranged from 317.66 to 334.33 μg mycelium/g soil and in the third layer was the same antigen amounts in all samples with about 367.66 μg mycelium/g soil.

The differences between the layers in this field are significant between the first and each of second and third layer ($P = 0.028$ and < 0.0001 , respectively), while it was ($P < 0.0001$) between the second and third layer.

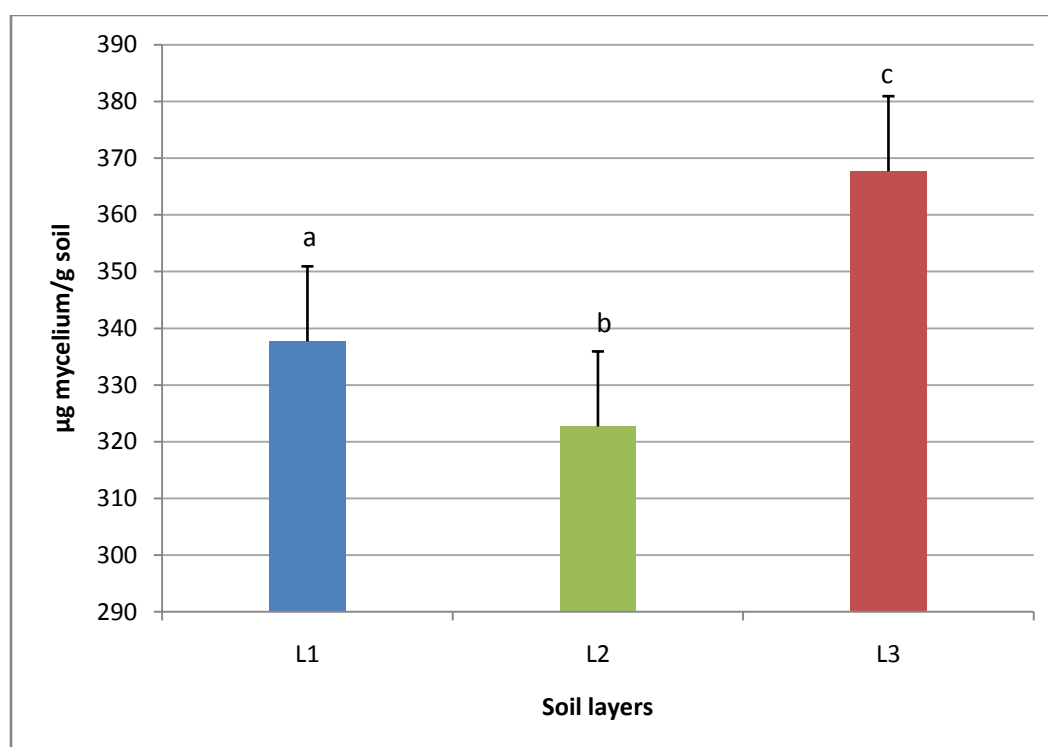


Fig. 49: The average antigen amounts (mean of $n=10$ soil samples) of Lower Saxonian field 1 using ELISA. Error bars indicate standard error (Tukey test, $P < 0.05$).

3.6.2.7 Quantification of *C. beticola* antigen in Lower Saxonian field 2

This field was cultivated with sugar beet 2 years before sampling date. The average antigen amounts of each layer in this field showed (Fig. 50 and Table A21) that the first layer contained the highest-antigen amount with 457.666 μg mycelium/g soil on average, followed by the second and third layers with 409.333 and 392.666 μg mycelium/g soil, respectively. The antigen amount in the first layer was 417.66 μg mycelium/g soil only in samples 1 and 4, while in the other samples was the same amount with about 467.66 μg mycelium/g soil. The second layer ranged from 384.33 to 434.33 μg mycelium/g soil and the third layer from 384.33 to 417.66 μg mycelium/g soil.

The differences between the layers in this field are significant between the first and each of second and third layer ($P < 0.0001$), while it was ($P = 0.014$) between the second and third layer.

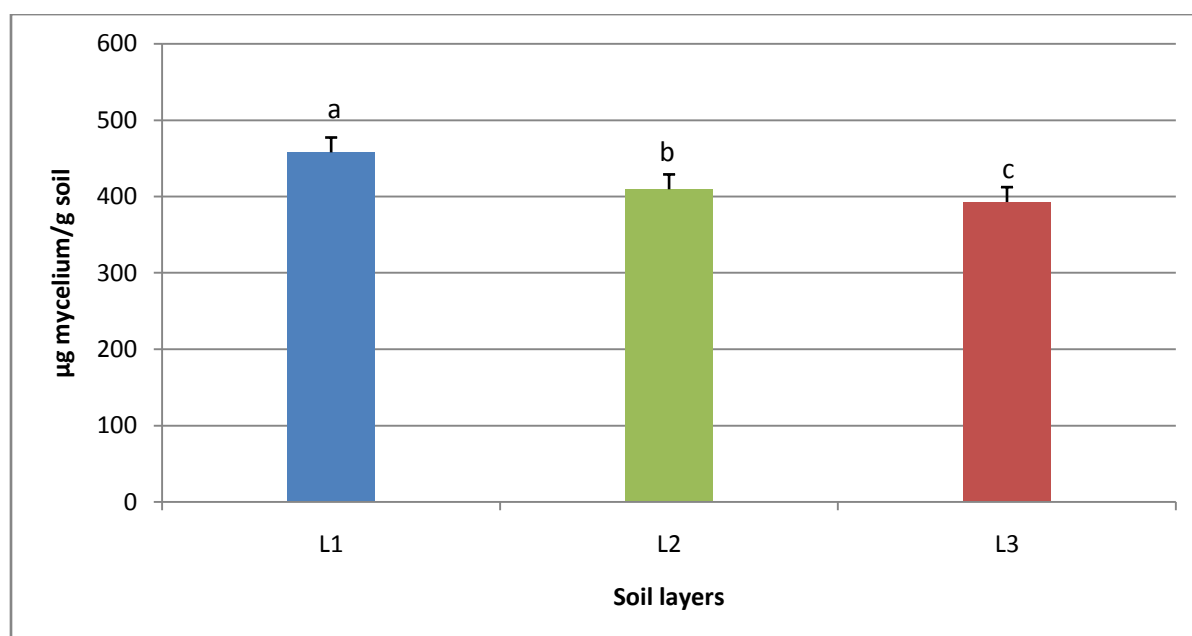


Fig. 50: The average antigen amounts (mean of $n=10$ soil samples) of Lower Saxonian field 2 using ELISA. Error bars indicate standard error (Tukey test, $P<0.05$).

3.6.2.8 Quantification of *C. beticola* antigen in Lower Saxonian field 3

This field was cultivated with sugar beet 1 year before sampling date. The average antigen amounts of each layer in this field showed (Fig. 51 and Table A22) that the first layer contained the highest-antigen amount with 539.333 μg mycelium/g soil on average, followed by the second and third layers with 491 and 451 μg mycelium/g soil, respectively. The antigen amount in the first layer was ranged from 501 to 567.66 μg mycelium/g soil, while in the second layer ranged from 484.33 to 501 μg mycelium/g soil and in the third layer was the same in all samples with about 451 μg mycelium/g soil.

The differences between the layers in this field are significant ($P < 0.0001$).

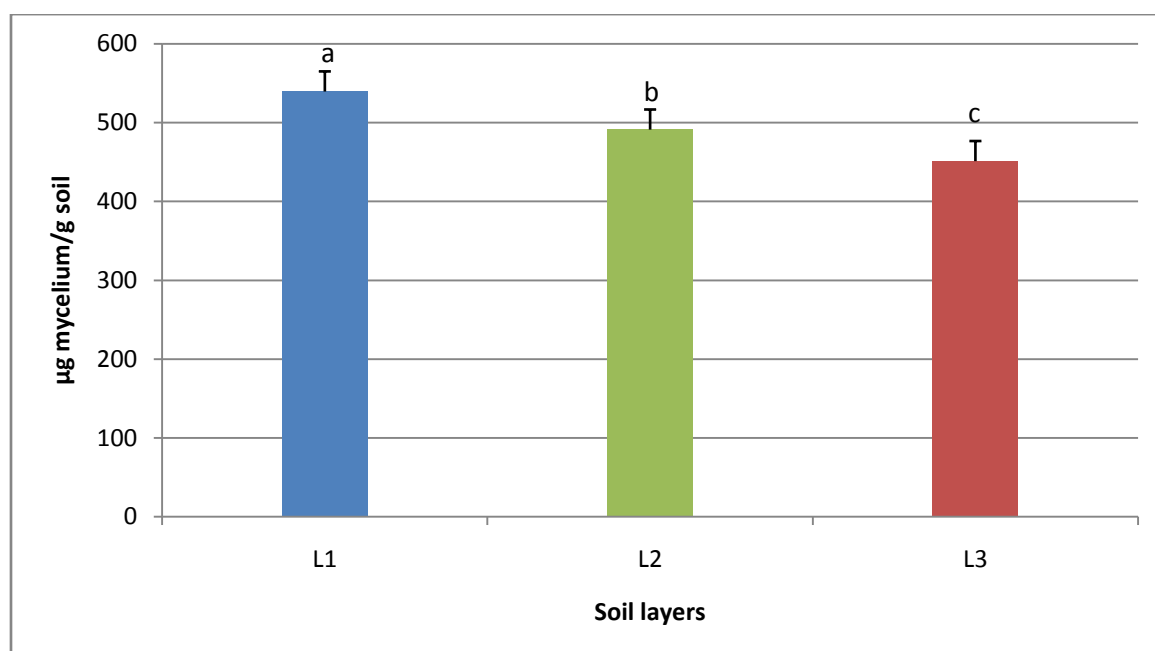


Fig. 51: The average antigen amounts (mean of $n=10$ soil samples) of Lower Saxonian field 3 using ELISA. Error bars indicate standard error (Tukey test, $P < 0.05$).

3.6.2.9 Quantification of *C. beticola* antigen in Lower Saxonian field 4

This field was cultivated with sugar beet 3 years before sampling date. The average antigen amounts of each layer in this field showed (Fig. 52 and Table A23) that the third layer contained the highest-antigen amount with 367.666 μg mycelium/g soil on average, followed by the first and second layers with 332.666 and 324.333 μg mycelium/g soil, respectively. The antigen amount in the first layer was ranged from 317.66 to 351 μg mycelium/g soil and the same range was observed in the second layer, while in the third layer was the same in all samples with about 367.66 μg mycelium/g soil.

The differences between the layers in this field are significant between each of the first and third layer and between the second and third layer ($P < 0.0001$), while it is not significant between the first and second layer ($P = 0.22$).

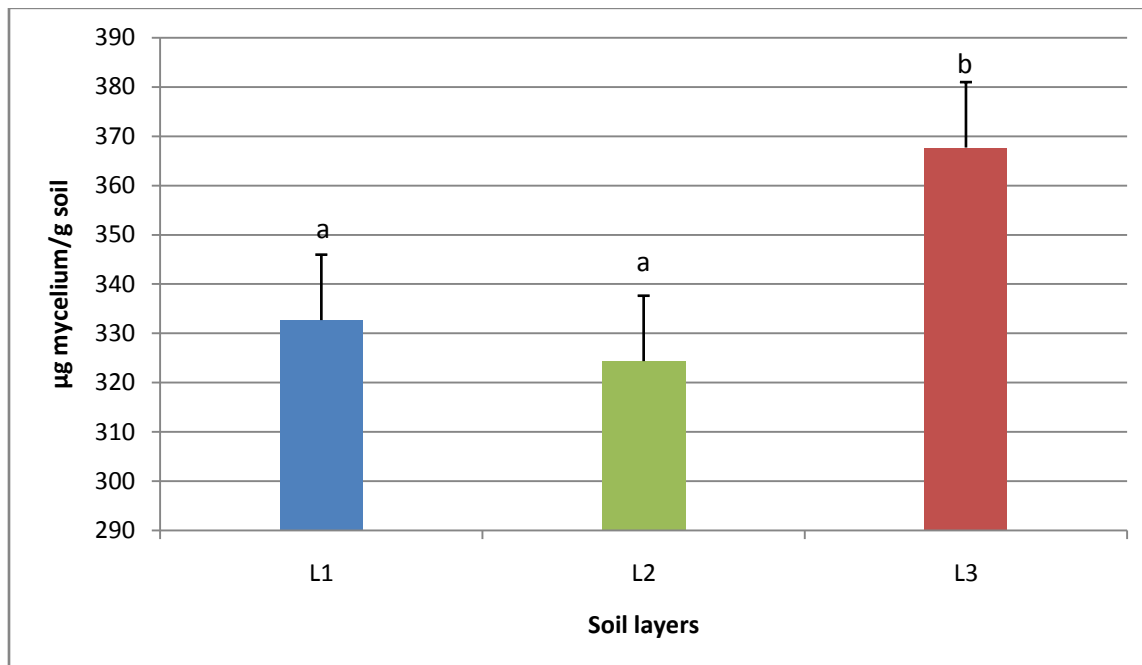


Fig. 52: The average antigen amounts (mean of $n=10$ soil samples) of Lower Saxonian field 4 using ELISA. Error bars indicate standard error (Tukey test, $P < 0.05$).

3.6.2.10 Quantification of *C. beticola* antigen in Lower Saxonian field 5

This field was cultivated with sugar beet 1 year before sampling date. The average antigen amounts of each layer in this field showed (Fig. 53 and Table A24) that the first layer contained the highest-antigen amount with 526 μg mycelium/g soil on average, followed by the second and third layers with 432.666 and 356 μg mycelium/g soil, respectively. The antigen amount in the first layer ranged from 501 to 534.33 μg mycelium/g soil and in the second layer from 417.66 to 467.66, while in the third layer was the same in all samples with about 351 μg mycelium/g soil except in the first sample which contained 401 μg mycelium/g soil.

The differences between the layers in this field are significant ($P < 0.0001$).

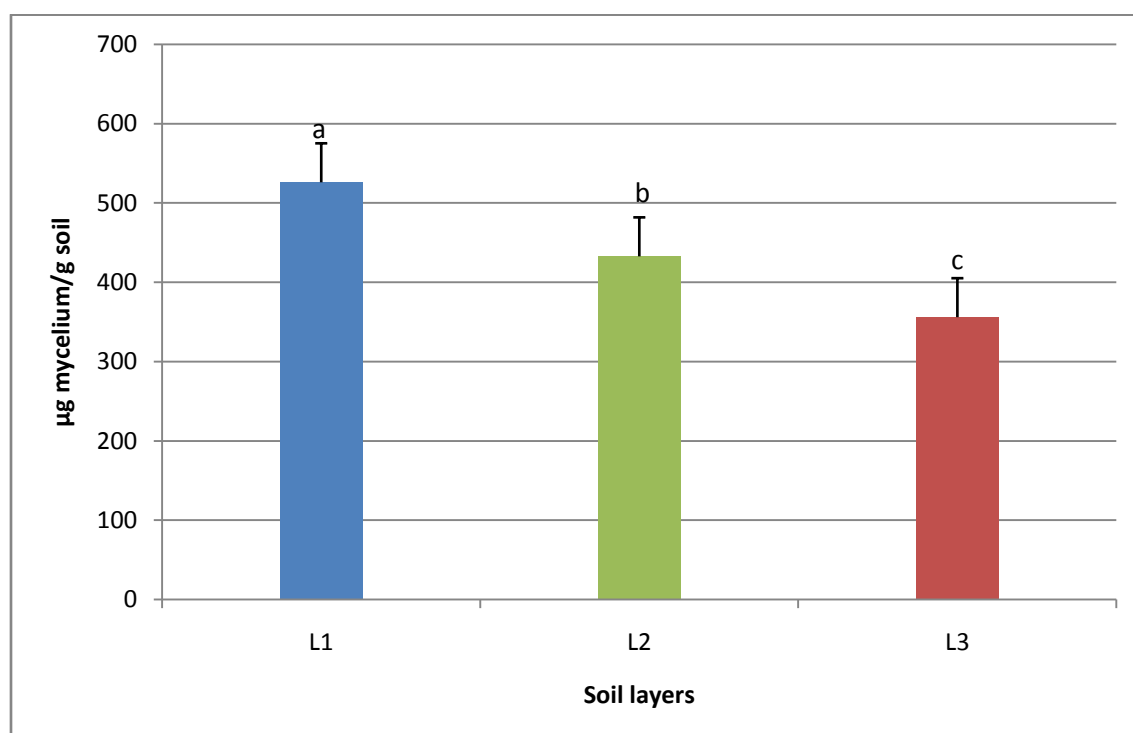


Fig. 53: The average antigen amounts (mean of $n=10$ soil samples) of Lower Saxonian field 5 using ELISA. Error bars indicate standard error (Tukey test, $P < 0.05$).

3.6.2.11 Quantification of *C. beticola* antigen in Lower Saxonian field 6

This field was cultivated with sugar beet 3 years before sampling date. The average antigen amounts of each layer in this field showed (Fig. 54 and Table A25) that the third layer contained the highest-antigen amount with 234.333 μg mycelium/g soil on average, followed by the second and first layers with 232.633 and 192.666 μg mycelium/g soil, respectively. The antigen amount in the first layer ranged from 167.66 to 201 μg mycelium/g soil and in the second layer from 217.66 to 251, while in the third layer was the same in all samples with about 234.33 μg mycelium/g soil.

The differences between the layers in this field are significant between each of the first and second layer and between the first and third layer ($P < 0.0001$), while it is not significant between the second and third layer ($P = 0.80$).

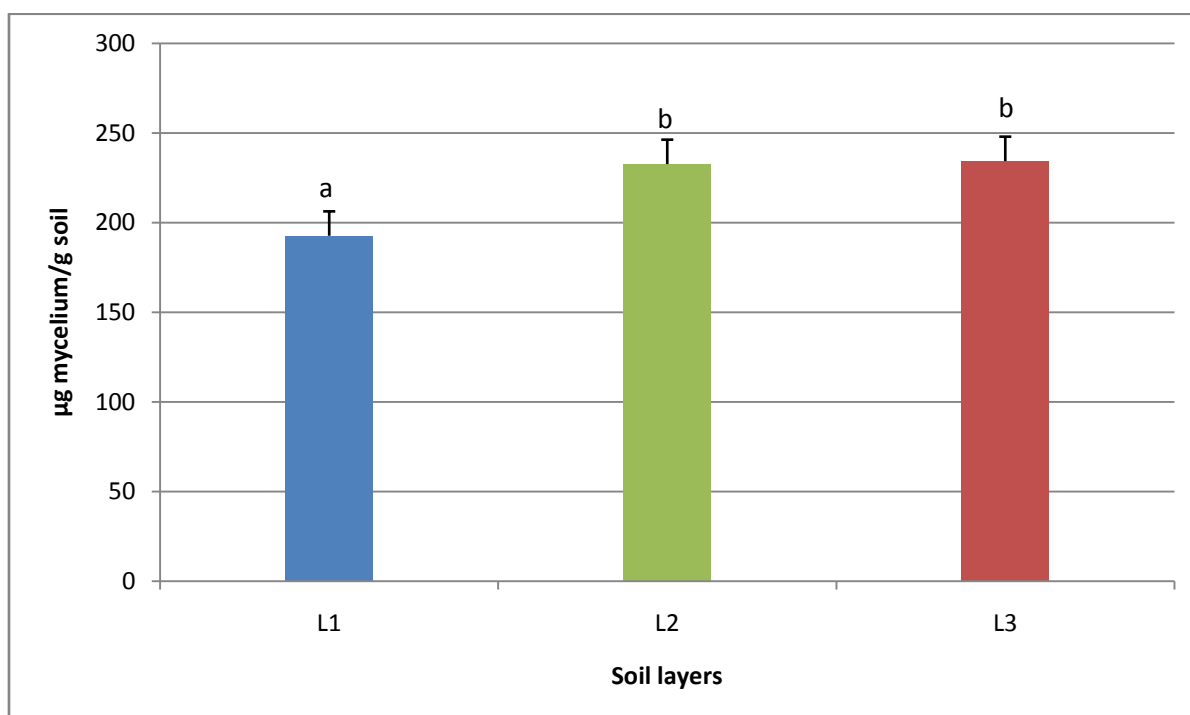


Fig. 54: The average antigen amounts (mean of $n=10$ soil samples) of Lower Saxonian field 6 using ELISA. Error bars indicate standard error (Tukey test, $P < 0.05$).

3.6.2.12 Comparison between the six Lower Saxonian fields

The comparison showed that the field contained the highest amounts of inoculum antigen was field 3 with a total amount of antigen with 14813.33 μg mycelium/g soil (which had been cultivated with sugar beet 1 year before sampling date), followed by field 5 with a total amount of antigen with 13146.67 μg mycelium/ g soil (which had been cultivated with sugar beet 1 year before sampling date). Field 2 came in the third order with a total amount of antigen with 12596.67 μg mycelium/g soil (which had been cultivated with sugar beet 2 years before sampling date), followed by field 1 with a total amount of antigen with 10280 μg mycelium/g soil (which had been cultivated with sugar beet 3 years before sampling date). Field 4 came in the fifth order with a total amount of antigen with 10246.67 μg mycelium/ g soil (which had been cultivated with sugar beet 3 years before sampling date). The field contained the lowest amounts of inoculum antigen was field 6 with a total amount of antigen with 6596.66 μg mycelium/g soil (which had been cultivated 3 years before sampling date) (Fig. 55).

The differences between all Lower Saxonian fields are significant ($P < 0.0001$) except between field 1 and field 4 ($P = 0.77$).

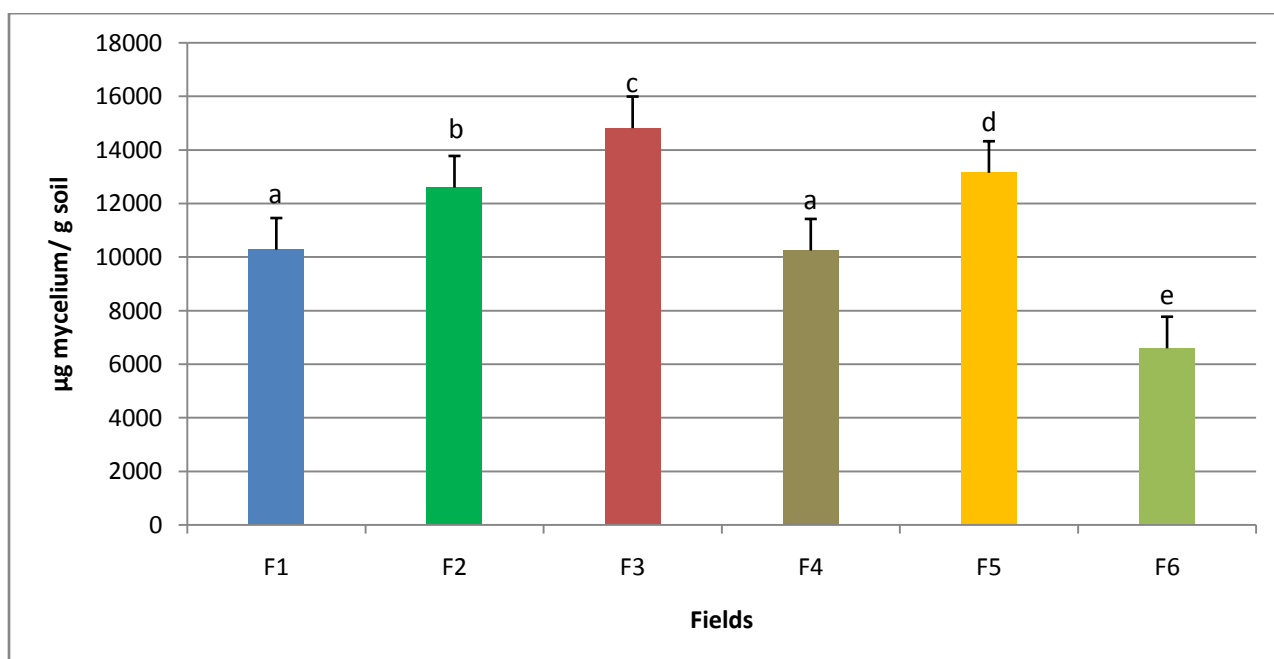


Fig. 55: Comparison of antigen amounts between the sixth Lower Saxonian fields. Error bars indicate standard error (Tukey test, $P < 0.05$).

3.6.2.13 Quantification of *C. beticola* antigen in USA field 1

This field was cultivated with sugar beet 2 years before sampling date and it maintained with conventional tillage after sugar beet cultivation. One sample was collected from each layer in this field. The results (Fig. 56 and Table A26) show that the first and second layers contained higher amounts of antigen than the third layer, where the antigen amounts in the first and second layers was 217.66 μg mycelium/g soil, while in the third layer was 201 μg mycelium/g soil.

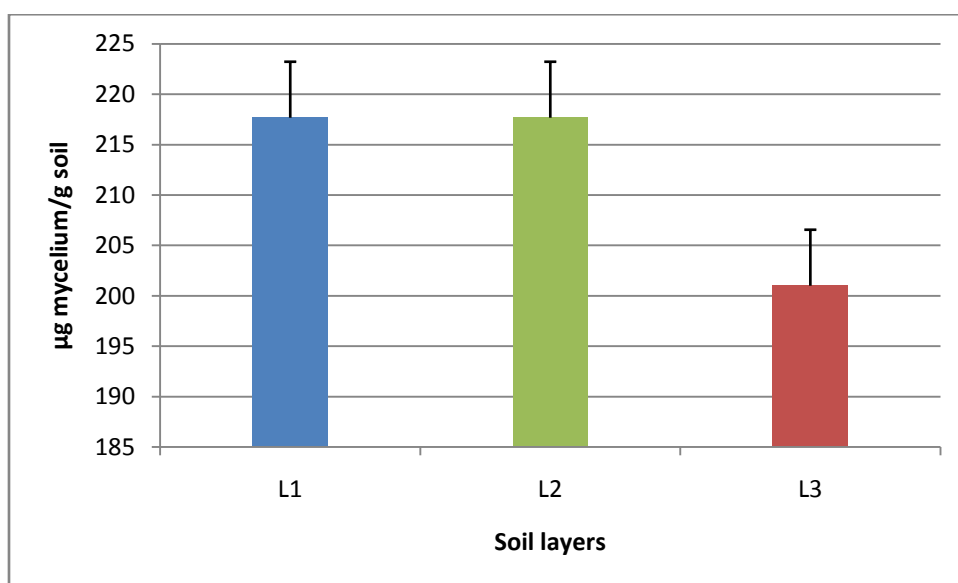


Fig. 56: Quantification of antigen amounts of USA field 1 ELISA. Error bars indicate standard error.

3.6.2.14 Quantification of *C. beticola* antigen in USA field 2

This field was cultivated with sugar beet 2 years before sampling date and it maintained with strip tillage after sugar beet cultivation. One sample was collected from each layer in this field. The results show (Fig. 57 and Table A27) that the three layers contained the same amounts of inoculum antigen with 201 μg mycelium/g soil.

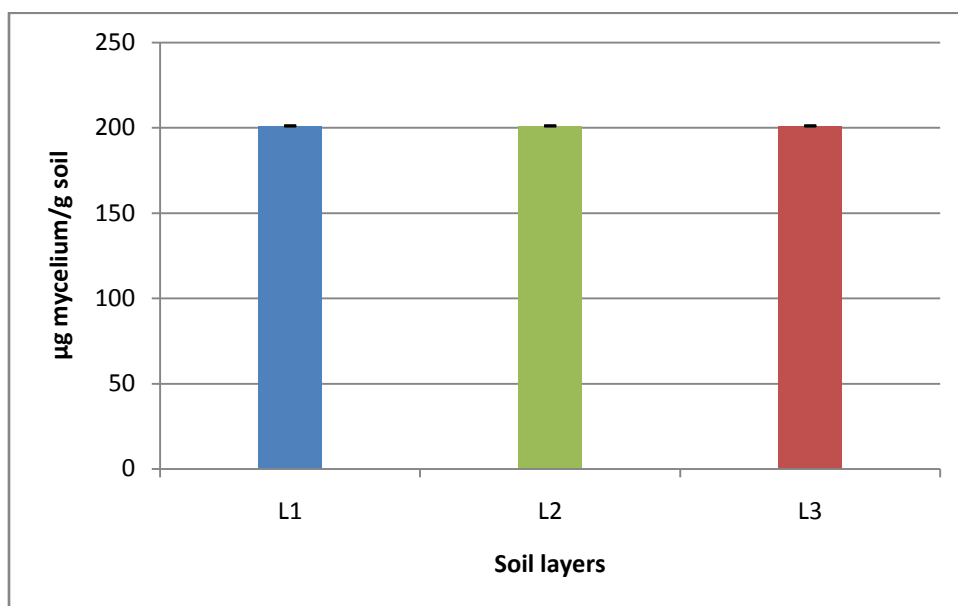


Fig. 57: Quantification of antigen amounts of USA field 2 using ELISA. Error bars indicate standard error.

3.6.2.15 Comparison between the two USA fields

The amount of antigen of the two USA fields samples were compared with each other. The results showed that field 1, which contained a total amount of antigen of 636.33 µg mycelium/g soil, was higher than field 2, which contained a total amount of antigen of 603 µg mycelium/g soil (Fig. 58).

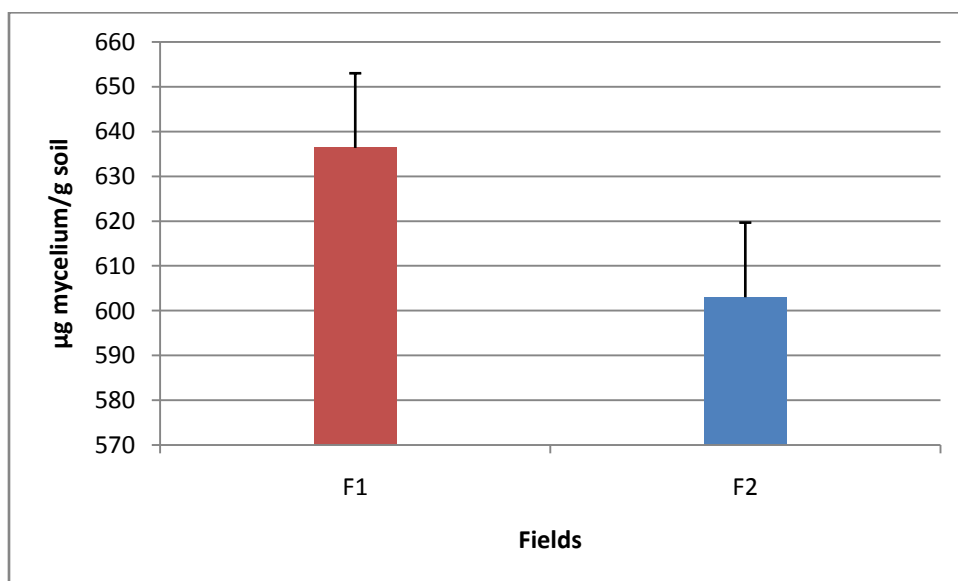


Fig. 58: Comparison of antigen amounts between the two USA fields. Error bars indicate standard error.

4 Discussion

Early inoculum detection methods are considered to be important tools for the control of plant pathogens. Unfortunately, management systems of most plant pathogens do not consider the factor “inoculum in the soil” due to lack of detection methods. For example, the IPM sugar beet model includes a prediction of the disease onset, which is due to missing detection methods defined as a “negative prognosis” and, therefore conversely, the disease free period is predicted. The reasons for the missing consideration of soil inoculum may be the difficulty of dealing with soil samples and getting a method for specific detection of the plant pathogen, as well as quantification in addition; in terms of serological methods, the constraints extracting the antigens from soil (Caesar *et al.* 2007).

In addition to the difficulties of antigen extraction from soil, the complex matrix of soil includes a variety of substances that inhibit the activity of polymerases and can affect the extracted DNA (Steffan *et al.* 1988; Demeke and Adams 1992). Many studies reported that soil samples are problematic for PCR assays due to the presence of inhibitory compounds such as humic and fulvic acids that inhibit *Taq* DNA polymerase through chelation of Mg^{2+} ions (Tsai and Olson 1991; Tebbe and Vahjen 1993).

C. beticola which causes *Cercospora* leaf spot (CLS) in sugar beet, is considered to be the most common and destructive foliar disease in sugar beet growing areas worldwide. The fungus can survive as pseudostromata for 1-2 years (Pool and McKay 1916; McKay and Pool 1918; Canova 1959). Therefore, early detection is of great significance for the efficient management of this pathogen. In this study, PCR and ELISA assays were successfully applied to detect and quantify *C. beticola* in soil.

Two specific primer sets with high specificity and sensitivity were developed and efficiently used for detection of *C. beticola* in soil using conventional PCR. One specific primer set, ITS3 /ITS4, was used for quantitative detection of *C. beticola* in soil using real-time PCR for its high specificity and sensitivity detection. ELISA also was successfully used for the detection of *C. beticola*, and 18 clones of monoclonal antibodies with a high specificity for *C. beticola* cell wall were developed. The clone 103 was used in all serological detection experiments because of its high detection specificity for *C. beticola*.

4.1 Development of specific primer sets and tests of sensitivity

4.1.1 Design of primer sets and their specificity test

The high specificity and the ability to distinguish closely related organisms is considered to be the most important advantage that molecular based detection methods have compared to conventional diagnostics. The specificity of PCR, be it conventional or real-time, depends upon the designing of proper PCR primers that are unique to the target organism. Thus, the designed primers must be of high quality for successful PCR amplification and detection of the target sequence. Additionally, non-specific amplification and/or primer-dimer formation could lead to a little or no product which is caused by the poorly designed primer (Dieffenbach *et al.* 1995).

Many factors can define the ability of an oligonucleotide to be used as a primer; (i) the association and dissociation between the primer and template at the annealing and extension temperatures, (ii) the stability degree of mismatched nucleotides at their locations and (iii) the efficiency of the taq polymerase enzyme to recognize and extend mismatched nucleotides.

Many professional web-based resources and software packages for PCR and primer design are available (Abd-Elsalam 2003). The internet program primer3 (<http://frodo.wi.mit.edu/primer3/input.htm>) was used to design all the primers which have been used in this study.

The aim was to develop a specific primer which could be used for the qualitative and quantitative detection of *C. beticola* in field soil using real-time PCR. For this purpose, specificity tests of the primer sets Cb-actinF1 /Cb-actinR2 , Cyt-F /Cyt-R and ITS3 /ITS4 were applied to *C. beticola* and 28 other fungal pathogens. Among these primer sets, Cyt-F /Cyt-R produced one slight fragment from *F. oxysporum* f.sp. *vasinfectum*, while the other two primer sets were highly specific and amplified one fragment from *C. beticola* but not any from the other 28 fungal pathogens tested.

In a previous study, Lartey *et al.* (2007), presented a specific primer set CBACTIN959L / CBACTN959R which had been designed from the actin gene sequence of *C. beticola*. This primer set amplified a high-size fragment of 959-bp. The authors surveyed several fields in the Lower Yellowstone River Valley (Eastern Montana and Western North Dakota) for the occurrence of *C. beticola*. The fields were either under sugar beet cultivation or in rotation with other crops. The results of this survey provided evidence for an extensive presence of *C.*

beticola in the tested fields. However, the large size of the primer (959-bp), made it not suitable for the quantitative detection by real-time PCR. In our study, the primer set ITS3 /ITS4 has a high level of specificity for *C. beticola* detection. Moreover, it amplified one fragment with a small size, 223-bp, and therefore, was ensuring its suitability for quantitative detection by real-time PCR.

Groenewald *et al.* (2008) analyzed the population structure and genotype diversity of 250 *C. beticola* isolates collected from different locations and proved that *C. beticola* is a pathogen with high genetic variation. *C. beticola* isolates sometimes differ by their tolerance to fungicides (Karaoglanidis *et al.* 2000; Weiland and Koch 2004). In another study, Turgay *et al.* (2010) identified different *C. beticola* pathotypes using pathogenicity tests with 100 isolates of *C. beticola*. These studies indicated that *C. beticola* is a pathogen with high genetic diversity. Therefore, two primer sets, Cb-actinF1 /Cb-actinR2 and ITS3 /ITS4 were tested for their specificity to detect 25 *C. beticola* isolates from Germany, Egypt and USA. Both primer sets amplified an expected PCR product from all 25 isolates tested, with the exception of isolate C2 which produced one slight fragment using the primer set Cb-actinF1 /Cb-actinR2. It is suggested that the two primer sets can be used for the detection of various *C. beticola* isolates.

In conclusion, the two primer sets developed in this study amplified a target fragment only from *C. beticola* but not any from the other 28 fungal pathogens. Furthermore, they amplified the same target fragment from 25 *C. beticola* isolates, which proved that the two developed primer sets can be used for detection of *C. beticola* in soil.

4.1.2 The sensitivity test of developed primer sets using conventional PCR

Diagnostic sensitivity is defined as a measure of the degree to detect the target pathogen in the sample. In case of lower sensitivity, false negative responses may be the consequence (Malorny *et al.* 2003). Thus, a high degree of diagnostic accuracy is characterized by the ability to detect, true and precisely the target micro-organism from a sample without interference from non target components. The high degree of sensitivity of molecular methods made presymptomatic detection and quantification of pathogens possible.

Many factors can affect the sensitivity of PCR, such as the nature of target sequences and primers as well as PCR conditions. Therefore, ten different dilutions of *C. beticola* DNA were prepared to check the sensitivity of designed primers and determine their detection limit at 28, 35 and 40 PCR cycles. The tests revealed a higher detection sensitivity of the primer set ITS3/ITS4 compared to the primer set Cb-actinF1/ Cb-actinR2, since it could detect as little as 100 pg, 5 pg and 0.5 pg at 28, 35 and 40 cycles, respectively (Fig. 14 A-F). The difference between the two genes used for the design of the two primer sets ITS3/ ITS4 and Cb-actinF1/ Cb-actinR2, rDNA and actin gene, respectively, may be the reason for the difference in detection limits.

4.2 Monoclonal antibody production and their specificity test

Monoclonal antibodies are considered to be the most accurate application of serological detection methods, which have a high degree of target specificity. This technique allows the selection of specific target epitopes to avoid “false positives”, unlike polyclonal antibodies, which recognize multiple epitopes of the pathogen. The hybridoma technology introduced by Köhler and Milstein (1975) is considered to be a revolutionary advancement in the process of antibody production that avoids many problems associated with the use of polyclonal antibodies. The principal advantage of hybridoma technology is the possibility of secreting and getting continuous supply of monoclonal antibodies by hybridomas, which are obtained by fusion of β -lymphocytes (antibody producing cells) and myeloma cells (capable of multiplying forever). Each hybridoma clone can produce identical antibodies that are specific for a single epitope of the antigen. In our study, the method of Köhler and Milstein (1975) was used to produce a monoclonal antibody against the cell wall of *C. beticola*. With this method, 18 positive clones were obtained which were tested after 1 week for reactivity with *C. beticola* and other fungal species *Chaetomium sp.*, *Fusarium acufiformis*, *F. sporotrichioides*, *Phytophthora infestans* and *Rhizoctonia solani*. The results indicated that all clones were specific for the detection of *C. beticola*, however, the OD values obtained by these clones varied. It is clear that the different hybridoma clones recognize the antigens with different affinities, which depend on the level of the binding and attraction between the antigen and antibody, so that they react with antigen at different OD values.

4.3 Determination of detection limit in soil probes by PCR and ELISA

4.3.1 Determination of detection limit in soil probes by PCR

Most soils and natural waters contain an abundance of tannic, humic and fulvic acids which bind to target nucleic acids and PCR primers. Therefore, they may inhibit many DNA polymerases (Abu Al-Soud and Rådström 2000; Kuske *et al.* 1998). These humic substances are mixtures of polyphenolics which are produced during the decomposition of organic matter. During extraction from soil they co-purify with DNA, water and plant material making problems with the extraction of DNA from soil (Kreader 1996). Such compounds limit the full potential of diagnostic PCR in plant pathology as they decrease amplification efficiency (Abu Al-Soud and Rådström 2000; Goodyear *et al.* 1994; Kong *et al.* 2003). The DNA is extracted from soil in the present study with a high degree of purity which allows using it in conventional or real-time PCR.

To determine the detection limit of the primer sets ITS3/ ITS4 and Cb-actinF1/ Cb-actinR2, two different inoculum sources were used, *C. beticola* mycelium and *C. beticola*-inoculated sugar beet leaves. From both inoculum sources, different dilutions were prepared to determine the detection limit. In both inoculum sources, the comparison between the two primer sets indicated that the primer set ITS3/ ITS4 had a higher level of detection than the primer set Cb-actinF1/ Cb-actinR2. These results were expected, depending on the PCR sensitivity test for the two primer sets, where the primer set ITS3/ ITS4 could detect a little amount of *C. beticola* inoculum with a higher efficiency than the primer set Cb-actinF1/ Cb-actinR2. This high degree of detection gives the primer set ITS3/ ITS4 the priority for detection of *C. beticola* inoculum in soil, where the amount of inoculum is unknown and depends on the environmental conditions which affect the survival of the pathogen in soil.

In spite of the high detection ability of the primer set ITS3/ ITS4 using mycelium as inoculum, the degree of detection was decreased when *C. beticola*-inoculated sugar beet leaves were applied as inoculum. This evidence could be explained by the different *C. beticola* growth between the two sources of inoculum, which is highest in its mycelium.

4.3.2 Determination of detection limit in soil probes by ELISA

In this experiment, ELISA assays were used to define the detection limit of two different inoculum sources, *C. beticola* mycelium and *C. beticola*-inoculated sugar beet leaves. The same dilutions of inoculum were tested as mentioned above. Compared to the PCR-experiment, similar results were obtained by ELISA, where the limit to detect the *C. beticola* antigen using the mycelium was higher than using inoculated sugar beet leaves. The explanation of these results is discussed (in section 4.3.1).

Compared with the results obtained by conventional PCR, ELISA showed a lower detection limit. This may be caused by the different targets of both methods, as PCR detects DNA whereas ELISA detects the antigen in samples. Another reason may be the differences between the extraction processes of target in both methods. During the extraction process of ELISA, the antigen is exposed to a high temperature during the homogenization step, which may affect the amount of extracted antigen and lead to a lower detection limit. Another possibility is the nature of soil used for dilutions and its chemical components and pH, which may affect the yield of extracted antigen.

4.4 Soil inoculum degradation in climate chamber

4.4.1 Soil inoculum degradation in climate chamber analyzed using real-time PCR

Quantitative PCR is a commonly used method for measuring the concentration of a template DNA input, which allows the cycle-to-cycle monitoring of the amplified DNA (Higuchi *et al.* 1992). Real-time PCR has significant advantages, compared to conventional PCR, since it does not require post-amplification processing steps and thus greatly reduces time and labour, which allows a large-scale analysis (Schena *et al.* 2002; Abd-Elsalam 2003).

Q-PCR recently has been used in several studies to measure the abundance or survival of a fungus in soil. For instance, the method was used to measure the abundance of *Phytophthora nicotianae* in soil (Huang *et al.* 2010). The authors investigated soil samples from tobacco field sites in China for the presence of *P. nicotianae* and reported that real-time PCR was 104-105 times more sensitive than conventional PCR. Another study (Jiménez-Fernández *et al.* 2011) used Q-PCR to quantify *Fusarium oxysporum* f. sp. *ciceris* in plant and soil. The authors developed a Q-PCR protocol that allows quantifying *F. oxysporum* f. sp. *ciceris* DNA down to 1 pg in soil, as well as in the plant root and stem. Moreover, they used this method to

differentiate susceptible from resistant chickpea reactions to the pathogen at 15 days after sowing in artificially infested soil. Lievens *et al.* (2006) used Q-PCR to quantify the presence of a number of economically important fungal and oomycete tomato pathogens in biological samples. These authors demonstrated the feasibility of the technique to quantify pathogen biomass in complex biological samples.

Khan *et al.* (2008) studied the survival and dispersal of *C. beticola* in soil, where its longevity was studied over a 3-year period under field conditions. *C. beticola*-infected leaves were placed at depths of 0, 10, and 20 cm and retrieved after 10, 22, and 34 months. The authors reported that the survival of *C. beticola* inoculum declined with time and soil depth. The inoculum left on the soil surface, 0 cm in depth, survived the longest (22 months) compared with that buried at 10 cm (10 months) and 20 cm (10 months).

Up to date, quantitative PCR was not applied for detection of *C. beticola* in soil. In the present study we quantified the degradation of soil borne inoculum in climate chamber during 6 months using real-time PCR. The first and second dilutions of infected leave-soil mixes (infected leaves soil mix: soil =1:10 and 1:100, respectively) were used as inoculum source. The highest DNA amounts were obtained always from the first dilution with 781.0133 ng/g soil at the beginning of inoculation and 2.4726 ng/g soil after 6 months. Similar degradation of *C. beticola* inoculum was observed from the second dilution, where the inoculum degraded during the time from 10.444 ng/g soil at the beginning of inoculation to 0.708 ng/g soil after 6 months. These results obtained by real-time PCR are in accordance with the findings of Khan *et al.* (2008), as in our experiment the inoculum degraded over time in both dilutions (please see Figure 17 in results section).

4.4.2 Soil inoculum degradation in climate chamber analyzed using ELISA

Studies in the ecology and management of *C. beticola* in soil have been hampered by difficulties in detecting and quantifying the amount of inoculum of this pathogen. In particular, the detection of antigens in soil implicates difficulties in extracting fungal antigens from soil and, in particular, the interference with non-specific soil contaminants, which may lead to false detection by ELISA. The most important factor which can affect the successful application of the soil-based immunoassays is the ability to recover significant amounts of the antigen as far as present in soil. The other factors include the dynamics of antigen production by the target fungus, the degradation of the antigen in soil, and the relationship between

antigen secretion and fungal biomass. Each of these factors requires careful analysis and testing in the development of a robust assay for the quantification of soil-borne fungi (Dewey *et al.* 1996). Retention of the antigen may occur on components of the soil solid phase by a range of physicochemical processes including electrostatic bonding by ion exchange after protonation and by hydrogen bonding, in particular between N-H groups and organic compounds. Due to the previous difficulties, there are a few studies which used ELISA assay for detection of fungal pathogens and non-pathogens in soil such as *Phytophthora* (Klopmeyer *et al.* 1988), *Glomus* (Wright and Morton 1989), *Thanatephorus* (Dusunceli and Fox 1992) and *Rhizoctonia* (Thornton *et al.* 1993).

Caesar *et al.* (2007) applied ELISA assay to detect and quantify *C. beticola* in soil using polyclonal antibodies. They reported that amounts as small as 0.38 µg of freeze-dried *C. beticola* mycelia dispersed in carbonate buffer could be quantified. Subsequently, they applied the method to *C. beticola* in field soil.

The objective of our current study was to determine the degradation of *C. beticola* inoculum in soil in climate chamber during 6 months using monoclonal antibodies and ELISA assay. The same dilutions which have been analyzed by real-time PCR were used for ELISA analysis. The OD values (Optical density or the absorbance) in this experiment were transferred to amount of *C. beticola* mycelium as described in results (section 3.5.2). The results obtained by ELISA assay were in agreement with real-time PCR results, where a degradation of the inoculum was observed independent from the inoculum amount (Fig. 19 in results). The highest antigen amounts were obtained always from the first dilution, with 467.66 µg mycelium/g soil at the beginning of inoculation and 301 µg mycelium/g soil after 6 months. However, the degradation of *C. beticola* inoculum antigen was slower than DNA. The reason may be that the longevity of the antigen in soil is higher than its DNA. Caesar *et al.* (2007) analyzed soil samples from 0 to 8 cm depths (0 to 1 cm, layer 1; 1 to 4 cm, layer 2; 4 to 8 cm layer 3) using ELISA for *C. beticola* antigen. The authors reported that *C. beticola* antigens were not degraded in soil. They have a high stability, and a proportion of immobilized antigens can be extracted from soil and detected by ELISA.

4.5 Quantification of *C. beticola* inoculum from field soil

4.5.1 Quantification of *C. beticola* DNA from field soil using real-time PCR

After successful quantification of *C. beticola* inoculum in climate chamber using real-time PCR, the method was applied to field soil. Different locations and soil depths were under scope. The comparison between the four Bavarian fields showed that the highest amounts of *C. beticola* DNA were obtained from field 4, which had been cultivated with sugar beet 1 year before sampling date. In contrast, field 3 contained the lowest amounts of DNA, which had been cultivated with sugar beet 3 years before sampling date, compared with the other Bavarian fields. These results indicated that the *C. beticola* inoculum degraded during the time. Although the present study is considered to be the first report of quantification of *C. beticola* DNA in soil using real-time PCR, Khan *et al.* (2008) studied the survival and dispersal of *C. beticola* in soil. The present study agreed with the results of Khan *et al.* (2008), who reported that the inoculum of *C. beticola* declined during the time. The comparison of *C. beticola* inoculum DNA between the first and second survey in field 4 showed that the degradation of *C. beticola* inoculum DNA took place in the second and third layers. However, the average DNA amount of first layer, field 4, even increased from 19.6 ng/g to 73,7 ng/g soil after 1 year. The reason for that may refer to the soil maintenance, where the crop after at first sampling date was winter wheat; sugar beet debris just were incorporated in soil by a cultivator and therefore preferably remained on the soil surface (10-15 cm). The analysis included three layers at depth 0-5, 5-15 and 15-30 cm, respectively. Thus this is a possible explanation, since the cultivator turned the upper first layer with the second one, which is supported by increasing *C. beticola* inoculum DNA in the second layer at the first sampling date.

The comparison of long distant locations showed the Lower Saxonian fields containing lower amounts of DNA than the Bavarian fields. The reason for that could be explained by the differences of soil components, since the lower Saxonian fields may contain some compounds which can damage DNA in soil. Another possibility is the differences of soil preparations.

Also the epidemiological behavior is of importance. *Cercospora beticola* needs for epidemic behavior (progression) temperatures of ca. 22°C. The fungus is more concentrated to the continental climate (e.g. in Bavaria) compared to the maritime influenced regions like Lower Saxony, where *Cercospora* leaf spot is not dominating the disease spectrum. *Ramularia beticola* and powdery mildew (*Erysiphe betae*) are of more importance to the northern sugar

beet regions. The infection potential of *Cercospora* leaf spot is much higher in the southern growing regions with higher temperatures.

An earlier report (Aardema *et al.* 1983) has shown that soil components other than organic materials and clay minerals can bind DNA and retard its enzymatic degradation. This would allow the DNA segments in soil and/or water to remain for a relatively long time.

Cai *et al.* (2006) studied the adsorption, desorption, and degradation by nucleases of DNA on four different colloidal fractions from a Brown soil and clay minerals. The authors reported that the presence of soil colloids and minerals provided protection to DNA against degradation by DNase I, where a higher level of protection was found in organic clays compared to inorganic clays. Additionally, they reported that DNA was tightly adsorbed by H₂O₂-treated clays (inorganic clays) in comparison to organic clays. In conclusion, the authors reported that the presence of organic material in soil increased the stability and therefore, the yield of DNA.

If comparing the six Lower Saxonian fields, similar results for the Bavarian fields were obtained, where higher amounts of *C. beticola* DNA were obtained from the fields which had been cultivated 1 year before sampling (fields 3 and 5). However, field 2 which had been cultivated 2 years before sampling contained lower amounts of DNA (with a total amount of 6.07 ng/g soil) than the fields 1, 4 and 6 which had been cultivated 3 years before sampling (with a total amount of 40.71, 19.38 and 48.95 ng/g soil, respectively). The interpretation of this result becomes evident by the lower amounts of DNA in all Lower Saxonian fields compared to the Bavarian fields.

4.5.2 Quantification of *C. beticola* antigen from field soil using ELISA

Additionally to PCR analysis, the field soil samples were investigated by ELISA to compare the goodness and practical suitability of methods. The OD values of soil samples were transferred to µg mycelium/g soil as described in results (section 3.5.2).

Among the four Bavarian fields the highest amounts of *C. beticola* antigen were obtained from field 4, which had been cultivated with sugar beets 1 year before. In contrast, field 3 contained the lowest amounts of *C. beticola* antigen, which had been cultivated with sugar beet 3 years before sampling date, compared with the other Bavarian fields. These results

agreed with the results of real-time PCR and the results of Khan *et al.* (2008), which indicated that the *C. beticola* inoculum degraded over the time.

Similar results were obtained from the comparison between the six Lower Saxonian fields, where the highest amounts of *C. beticola* antigen were obtained from the fields which were cultivated with sugar beets 1 year before sampling date (field 3 and 5).

The real-time PCR and ELISA results of the Bavarian fields were similar to each other, where both of them proved the presence of *C. beticola* in the tested fields. However, the results obtained from real-time PCR of the Lower Saxonian fields revealed that these fields contained lower amounts of *C. beticola* DNA. In contrast, the results obtained by ELISA established high amounts of *C. beticola* antigen. The reason may be that the longevity of the antigen in soil is higher than its DNA and damages of DNA in these fields, as discussed before. These results were in agreement with the results obtained from the experiment of inoculum degradation in soil climate chamber, which indicated that the degradation of *C. beticola* inoculum antigen was slower than its DNA. Additionally, these results were in agreement with the study of Caesar *et al.* (2007) who designed (pAb) against the *C. beticola* cell wall. These authors reported that *C. beticola* antigens were not degraded in soil; therefore have a high stability and a proportion of immobilized antigens can be extracted from soil and detected by ELISA.

5 Summary

Cercospora leaf spot (CLS) is considered to be the most common and destructive foliar disease in sugar beets worldwide. It causes reductions in sugar yield and financial returns. It is caused by the fungus *Cercospora beticola* which survives as stromata on sugar beet leaf residues in soil. These stromata germinate under warm and moist conditions by producing conidia which are dispersed as primary inoculum to initiate infection of sugar beet leaves.

The objective of this research was to develop effective methods for qualitative and quantitative detection of *C. beticola* in soil. The soil borne inoculum is suggested to be an important factor for disease prediction. Therefore, these methods are aimed to improve integrated pest management systems (IPM) of sugar beet. In this study two diagnostic methods, polymerase chain reaction (PCR) and Enzyme linked immunoabsorbent assay (ELISA) technique were successfully applied for qualitative and quantitative detection of *C. beticola* in soil.

Identification of *C. beticola*-specific sequences was performed using the search on NCBI-GenBank web site, where three sequences were identified; a 469-bp rDNA sequence, a 967-bp cytochrome b (cytb) mRNA sequence and a 1195-bp actin gene. Three primer sets were then designed using the internet program primer3 from the sequences mentioned above; ITS3/ITS4, Cyt-F/ Cyt-R and Cb-actinF1/ Cb-actinR2, respectively. A primary specificity test for the three primer sets was carried out with three *C. beticola* isolates. The three primers sets amplified a single fragment each from the *C. beticola* isolates with different sizes; 798-bp for Cb-actinF1/ Cb-actinR2, 243-bp for Cyt-F/ Cyt-R and 223-bp for ITS3/ ITS4. The three primer sets were then tested for their specificity with 28 other fungal pathogens. Hence, the two primer sets, ITS3/ ITS4 and Cb-actinF1/ Cb-actinR2, were found highly specific since they amplified one single fragment only from *C. beticola* isolates but not from the 28 other fungal pathogens tested. The primer set Cyt-F/ Cyt-R also established a high specificity; exceptionally from *F. oxysporum* f.sp. *vasinfectum*, one slight band was amplified. The two primer sets; ITS3/ ITS4 and Cb-actinF1/ Cb-actinR2 were then tested for their specificity with 25 *C. beticola* isolates from Germany, Egypt and USA. The developed two primer sets amplified one strong fragment from all *C. beticola* isolates except one isolate (isolate C2)

which showed one slight fragment using the primer set Cb-actinF1/ Cb-actinR2. Three different cycle numbers of conventional PCR, 28, 35 and 40 cycles, were used for the sensitivity test of the developed two primer sets; ITS3/ ITS4 and Cb-actinF1/ Cb-actinR2. The primer set ITS3/ ITS4 showed a high level of detection since it could detect as little as 0.5 pg *C. beticola* genomic DNA, while the primer set Cb-actinF1/ Cb-actinR2 could detect up to 10 pg in a conventional PCR.

For the detection of *C. beticola* antigen, specific monoclonal antibodies (mAbs) were produced using the method of Köhler and Milstein (1975) at the Institute of Biochemistry, University of Kiel. Eighteen clones were obtained against *C. beticola* which were tested using ELISA assay for their specificity with *C. beticola* mycelium and the fungal species *Chaetomium sp.*, *Fusarium acufiformis*, *F. sporotrichioides*, *Phytophthora infestans* and *Rhizoctonia solani*. All antibody clones gave positive results only with *C. beticola* and negative results with the other fungal species indicating that these antibodies are specific for *C. beticola*.

ELISA and real-time PCR were used to determine the degradation of *C. beticola* inoculum in soil during 6 months under controlled conditions in climate chamber. *C. beticola* infected sugar beet leaves were mixed with soil at a 1:1 ratio and then two different dilutions with soil were prepared (infected leaves soil mix: soil =1:10 and 1:100). The two dilutions were then used as *C. beticola* inoculum in soil. Both detection methods showed degradation of *C. beticola* inoculum over time in both dilutions. However, ELISA revealed slower disappearance of *C. beticola* antigen in soil than DNA. The latter findings indicate that the antigen of the fungus persists in soil longer than DNA.

After developing and proving the detection methods under controlled conditions at known inoculum amounts, these methods were applied to soil samples from natural fields of different locations. The soil samples were collected from two regions in Germany, Bavaria and Lower Saxony and in addition from one location in Montana (USA). These fields were under different crop rotations and soil maintenance. The samples were taken from three different layers: 0-5 cm, 5-15 cm and 15-30 cm.

Additionally, different samples were collected 1 year after the first survey from the field which contained the biggest inoculum of *C. beticola*, field number 4 of Bavaria location. The objective of the second survey was to determine the degradation of *C. beticola* inoculum in field soil; therefore the samples were collected from the same area and in the same way as done in the first survey.

Amplicons derived from PCR using the primer set ITS3/ ITS4 were sequenced and compared to DNA sequence from pure culture *C. beticola*. Alignment of sequences of the amplified products confirmed them to be those of *C. beticola*.

The comparison between the surveyed fields, using the results obtained by real-time PCR, showed that the highest amounts of *C. beticola* DNA were obtained from the fields which were cultivated with sugar beet 1 year before sampling date. In contrast, the lowest amounts of *C. beticola* DNA were obtained from the fields which were cultivated with sugar beet 3 years before sampling date. However, the Lower Saxonian field 2 which was cultivated with sugar beet 2 years before sampling date showed the lowest amounts of *C. beticola* DNA compared with the other Lower Saxonian fields. The comparison between the first and second survey, 1 year after the first one, in the Bavarian field 4 showed that *C. beticola* inoculum DNA degraded in the second and third layers. However, the first layer in the second survey showed higher average amounts of DNA than at the first sampling date which may refer to the soil maintenance.

Similar results were obtained using ELISA assay, where the highest amounts of *C. beticola* antigen were obtained from the fields which were cultivated with sugar beet 1 year before sampling date. In contrast, the lowest amounts of *C. beticola* antigen were obtained from the fields which were cultivated with sugar beet 3 years before sampling date.

6 Zusammenfassung

Die *Cercospora*-Blattfleckenkrankheit (CLS) gilt als die häufigste und destruktivste Blattkrankheit im Zuckerrübenanbau weltweit. Sie verursacht eine Senkung des Zuckerertrages sowie der finanziellen Renditen. Die Krankheit wird verursacht durch den pilzlichen Schaderreger *Cercospora beticola*, welcher als Stroma auf Zuckerrübenblattresten im Boden überlebt. Diese Stroma bilden unter warmen und feuchten Bedingungen eine Vielzahl büschelartiger angeordneter Konidienträger mit daran befindlichen Konidien aus. Diese Konidien stellen das primäre Inokulum für eine Infektion der Zuckerrübenblätter dar.

Das Ziel dieser Arbeit war es, effektive Methoden zum qualitativen und quantitativen Nachweis von *C. beticola* im Boden zu entwickeln, da das bodenbürtige Inokulum einen wichtigen Faktor für die Vorhersage dieser Krankheit darstellt. Mit Hilfe dieser Methoden soll das Integrierte Pflanzenschutz Management (IPM) der Zuckerrübe verbessert werden. In dieser Arbeit wurden zwei diagnostische Methoden, die Polymerase-Kettenreaktion (PCR) sowie ELISA-Technik, erfolgreich für den qualitativen und quantitativen Nachweis von *C. beticola* im Boden angewendet.

Die Identifizierung von *C. beticola*-spezifischen Sequenzen erfolgte mit Hilfe der NCBI-GenBank-Website, auf der drei Sequenzen identifiziert wurden. Eine 469-bp rDNA-Sequenz, eine 967-bp Cytochrom b (cytb) mRNA-Sequenz und ein 1195-bp-Aktin Gen. Drei Primer-Sets wurden basierend auf diesen Sequenzen mit Hilfe des Internetprogramms Primer 3 entwickelt: ITS3/ITS4, Cyt-F/Cyt-R und Cb-actinF1/Cb-actinR2. Ein primärer Spezifitätstest für diese drei Primer-Paare wurde mit drei *C. beticola*-Isolaten durchgeführt. Die drei Primer-Paare amplifizierten jeweils ein Fragment aus den *C. beticola*-Isolaten mit verschiedenen Fragmentgrößen: 798 bp für Cb-actinF1/Cb-actinR2, 243 bp für Cyt-F/Cyt-R und 223 bp für ITS3/ITS4. Die drei Primer-Sets wurden dann auf ihre Spezifität untersucht und mit 28 anderen pilzlichen Krankheitserregern kreuzgetestet. Die beiden Primer-Paare ITS3/ITS4 und Cb-actinF1/Cb-actinR2 waren sehr spezifisch, da sie nur ein einziges Fragment der *C. beticola*-Isolate amplifizierten, nicht aber von den anderen 28 getesteten pilzlichen Pathogenen. Das Primer-Paar Cyt-F/Cyt-R zeigte ebenfalls eine hohe Spezifität, jedoch wurde auch bei *F. oxysporum f. sp. vasinfectum* ein PCR-Produkt amplifiziert. Die beiden Primer-Sets ITS3/ITS4 und Cb-actinF1/Cb-actinR2 wurden dann auf ihre Spezifität mit 25 *C. beticola*-Isolaten aus Deutschland, Ägypten und den USA getestet. Die zwei entwickelten

Primer-Sets amplifizierten jeweils ein deutliches Amplifikat aus allen *C. beticola*-Isolaten. Lediglich ein Isolat (Isolat C2) zeigte unter Verwendung des Primer-Sets Cb-actinF1/Cb-actinR2 eine schwache Bande. Für den Sensitivitätstest der zwei entwickelten Primer-Paare wurden drei verschiedene Zyklenzahlen für die konventionelle PCR verwendet: 28, 35 und 40 Zyklen. Das Primer-Paar ITS3/ITS4 zeigte eine hohe Empfindlichkeit der Detektion, da lediglich 0,5 pg genomischer DNA von *C. beticola* detektiert werden konnte, während mit dem Primer-Paar Cb-actinF1/Cb-actinR2 minimal 10 pg genomischer DNA mit der konventionellen PCR nachgewiesen wurden.

Für den Nachweis von *C. beticola* Antigenen wurden spezifische monoklonale Antikörper (mAk) unter Verwendung der Methode von Köhler und Milstein (1975) am Institut für Biochemie, Universität Kiel, hergestellt. Achtzehn Klone wurden auf ihre Spezifität mittels ELISA gegen *C. beticola* und einige andere Pilzarten (*Chaetomium* spp., *Fusarium acufiformis*, *F. sporotrichioides*, *Pytophthora infestans*, *Rhizoctonia solani*) getestet. Alle Antikörperklone zeigten lediglich positive Ergebnisse mit *C. beticola*, während die Ergebnisse mit den anderen getesteten Pilzspezies negativ waren, was auf die Spezifität dieser Antikörper für *C. beticola* hinweist.

ELISA und real-time PCR wurden verwendet, um den Abbau des *C. beticola*-Inokulums im Boden unter kontrollierten Bedingungen in der Klimakammer in einem Zeitraum von 6 Monaten zu erfassen. Mit *C. beticola* infizierte Zuckerrübenblätter wurden in einem Verhältnis von 1:1 mit Boden gemischt und anschließend zwei verschiedene Verdünnungen mit Erde hergestellt (infizierte Blätter Bodenmischung: Boden = 1:10 bzw. 1:100). Diese beiden Verdünnungen wurden dann als *C. beticola*-Inokulum im Boden verwendet. Mit beiden Nachweismethoden konnte der Abbau des *C. beticola*-Inokulums im Zeitverlauf in beiden Verdünnungen gezeigt werden, wobei jedoch mit dem ELISA-Test *C. beticola*-Antigene länger im Boden nachgewiesen werden konnte als die *C. beticola* DNA mittels PCR. Diese Befunde deuten darauf hin, dass die Antigene des Pilzes länger im Boden verbleiben als die DNA.

Nach der Entwicklung und Erprobung der Nachweismethoden unter kontrollierten Bedingungen mit bekannten Inokulum-Mengen, wurden diese Methoden für Bodenproben von verschiedenen Schlägen und Standorten angewendet. Die Bodenproben wurden aus zwei

Regionen in Deutschland, Bayern und Niedersachsen, sowie einer Region in Montana (USA) entnommen. Auf den beprobten Feldern wurden verschiedene Bodenbearbeitungsverfahren und Fruchtfolgen durchgeführt. Die Probenahme erfolgte aus drei verschiedenen Bodenschichten: 0-5 cm, 5-15 cm und 15-30 cm.

Der Vergleich zwischen den untersuchten Feldern basierend auf den Ergebnissen der real-time PCR zeigte, dass die höchsten Mengen an *C. beticola* DNA von den Feldern stammte, auf denen 1 Jahr vor der Probenahme Zuckerrüben angebaut wurden. Im Gegensatz dazu wurden die niedrigsten Mengen an *C. beticola* DNA von den Feldern nachgewiesen, auf denen 3 Jahre vor der Probenahme Zuckerrüben angebaut wurden. Allerdings konnte auf Feld 2 in Niedersachsen, auf welchem Zuckerrüben 2 Jahre vor der Probenahme angebaut wurden, die niedrigste Mengen an *C. beticola* DNA verglichen mit den anderen niedersächsischen Feldern gefunden werden. Der Vergleich zwischen der ersten und zweiten Untersuchung (1 Jahr nach dem ersten Jahr), die mit Bodenproben von Feld 4 aus Bayern durchgeführt wurden, zeigte, dass die *C. beticola* Inokulum DNA in der zweiten und dritten Bodenschicht degradiert war. Jedoch konnte in der ersten Schicht im zweiten Jahr durchschnittlich höhere Mengen an DNA als bei der ersten Probenahme ein Jahr zuvor detektiert werden.

Ähnliche Ergebnisse wurden mit dem ELISA-Test erzielt, bei denen die höchsten Mengen an *C. beticola*-Antigenen von den Feldern stammte, auf denen Zuckerrüben 1 Jahr vor der Probenahme angebaut wurden. Im Gegensatz dazu wurden die niedrigsten Mengen von *C. beticola*-Antigenen von Feldern gewonnen, auf denen Zuckerrüben 3 Jahre vor der Probenahme angebaut wurden.

7 References

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8. Appendix

8.1 Tables

Table A1 Quantification of *C. beticola* DNA in Bavarian field 1

No.	Layer1					Layer2					Layer3				
	R1	R2	R3	*M (ng/μl)	*M (ng/g soil)	R1	R2	R3	*M (ng/μl)	*M (ng/g soil)	R1	R2	R3	*M (ng/μl)	*M (ng/g soil)
1	0.09576	0.00195	0.00294	0.03355	13.42	0	0.002856	0	0	0	0.131833	0.104568	0.05595	0.09745	38.98
2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0	0	0.026731	0	0.00891	3.564
4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0	0	0.021445	0.2545	0.034312	0.10342	41.368
6	0.02830	0.00513	0.00860	0.01401	5.604	0	0	0	0	0	0.16953	0.2786	0.154758	0.20096	80.3852
7	0.0796	0.001670	0.00259	0.027953	11.1812	0	0.04442	0.00169	0.015678	6.2712	1.5132	0.34894	0.51147	0.79120	316.4812
8	0.07502	0.03280	0.01640	0.041407	16.5628	0	0.01908	0	0.00636	2.544	0.0266	0.033277	0.066469	0.04211	16.846
9	0.113501	0.19951	0.02829	0.113767	45.5068	0	0	0	0	0	0.677206	0.3445	0.014245	0.34531	138.1268
10	0	0.002114	0.002114	0.001409	0.5636	0	0	0	0	0	0.00488	0.05673	0.11278	0.05813	23.252
*M	9.28384					0.88152					65.90032				

*M: Mean

Table A2 Quantification of *C. beticola* DNA in Bavarian field 2

No.	Layer1					Layer2					Layer3				
	R1	R2	R3	*M (ng/μl)	*M (ng/g soil)	R1	R2	R3	*M (ng/μl)	*M (ng/g soil)	R1	R2	R3	*M (ng/μl)	*M (ng/g soil)
1	0.49037	0.42016	0.53994	0.48349	193.396	0	0.001201	0.00104	0.000747	0.2988	0.8770	0.00873	0.38485	0.423527	169.4108
2	0.79449	3.1339	0.61648	1.514957	605.9828	0.005934	0.06492	0.00614	0.025665	10.266	0	0	0	0	0
3	0.00135	0.02169	0.01989	0.01431	5.724	0.000239	0.00679	0.000706	0.002578	1.0312	0.001400	0.00932	0.009164	0.006628	2.6512
4	0.00169	0.02209	0.02164	0.01514	6.056	0.000182	0.00489	0.007530	0.004201	1.6804	0.00113	0.03085	0.000516	0.010832	4.3328
5	0.07487	0.76746	0.051130	0.29782	119.128	0.00219	0.01968	0.022598	0.014823	5.9292	0.00282	0.001740	0	0.00152	0.608
6	0.0669	0.15872	0.07478	0.100133	40.0532	0.0176	0.05843	0.05892	0.044983	17.9932	0.00170	0.27828	0.003341	0.09444	37.776
7	0.1224	0.21931	0.21539	0.1857	74.28	0.18522	0.9991	0.01948	0.401267	160.5068	0	0	0	0	0
8	0.25478	0.17249	0.22855	0.218607	87.4428	0.02539	0.12675	0.12799	0.093377	37.3508	0.001358	0.01561	0.02842	0.015129	6.0516
9	0.014528	0.14555	0.13118	0.097086	38.8344	0.02913	0.00149	0	0.010207	4.0828	0.00103	0.04415	0.02065	0.021943	8.7772
10	0.0418	0.20133	0.04227	0.095133	38.0532	0.26927	0.9778	0.24509	0.497387	198.9548	0.01340	0.00473	0	0.006043	2.4172
*M					120.895					43.8094					23.20248

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Table A3 Quantification of *C. beticola* DNA in Bavarian field 3

No.	Layer1					Layer2					Layer3				
	R1	R2	R3	*M (ng/μl)	*M (ng/g soil)	R1	R2	R3	*M (ng/μl)	*M (ng/g soil)	R1	R2	R3	*M (ng/μl)	*M (ng/g soil)
1	0.003797	0.03938	0.0427	0.028626	11.450	0	0.00685	0	0.002283	0.9132	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5	0.00262	0.00311	0.00209	0.002607	1.0428	0.00138	0	0	0	0	0	0	0	0	0
6	0.04214	0.03194	0.00368	0.02592	10.368	0.00286	0.00387	0	0.00243	0.972	0.00202	0.0014	0.00171	0.00171	0.684
7	0.00853	0.01066	0.00552	0.008237	3.2948	0	0	0.00177	0	0	0	0	0	0	0
8	0.003016	0	0.00445	0.002489	0.9956	0	0	0	0	0	0	0	0	0	0
9	0.00188	0.15017	0.00257	0.05154	20.616	0	0.00406	0	0.001353	0.5412	0	0	0	0	0
10	0	0.06830	0	0.022767	9.1068	0	0	0.00767	0.002557	1.0228	0	0	0	0	0
*M					5.6874					0.34492					0.0684

*M: Mean

Table A4 Quantification of *C. beticola* DNA in Bavarian field 4

No.	Layer1					Layer2					Layer3				
	R1	R2	R3	*M (ng/μl)	*M (ng/g soil)	R1	R2	R3	*M (ng/μl)	*M (ng/g soil)	R1	R2	R3	*M (ng/μl)	*M (ng/g soil)
1	0.010839	0.013994	0.0111	0.011978	4.7912	0.13137	0.013667	0.010222	0.051753	20.7012	0.001264	0.000923	0.07865	0.026946	10.7784
2	0.00569	0.002795	0.005235	0.004573	1.8292	0.09480	0.084123	0.0640945	0.081006	32.4024	0.14642	0.14642	0.224535	0.172458	68.9832
3	0.019502	0.01232	0.19063	0.074151	29.6604	0.002809	0.001285	0.001853	0.001982	0.7928	0.15843	0.15843	3.6335	1.316787	526.7148
4	0.01255	0.013933	0.03750	0.021328	8.5312	0.18018	0.24881	0.24757	0.22552	90.208	0.041644	0	0.01750	0.019715	7.886
5	0.25859	0.30238	0.24415	0.268373	107.3492	1.3193	3.6339	0.23792	1.730373	692.1492	0.16421	0.12211	0.6420	0.30944	123.776
6	0.057961	0.050837	0.051992	0.053597	21.4388	0.876	0.18833	0.18833	0.417553	167.0212	0.26277	0.226195	0.27809	0.255685	102.274
7	0.01856	0.004666	0.010405	0.01121	4.484	0.8089	0.34186	0.01562	0.388793	155.5172	1.1496	0.32794	0.29389	0.590477	236.1908
8	0.017857	0.016403	0.01859	0.017617	7.0468	4.5286	0.557612	0.557612	1.881275	752.51	0.68333	0.867946	0.942251	0.831176	332.4704
9	0.00784	0.00489	0.03759	0.016773	6.7092	0.118508	0.16505	0.13220	0.138586	55.4344	0.057532	0.002786	0.01404	0.024786	9.9144
10	0.012031	0.008842	0.01157	0.010814	4.3256	0.306109	0.16023	0.13783	0.20139	80.556	0.049535	0.03904	0.0787	0.055758	22.3032
*M					19.61656					204.7292					144.1291

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Table A5 Quantification of *C. beticola* DNA in Bavarian field 4. 1 year after the first sampling date

No.	Layer1					Layer2					Layer3				
	R1	R2	R3	*M (ng/μl)	*M (ng/g soil)	R1	R2	R3	*M (ng/μl)	*M (ng/g soil)	R1	R2	R3	*M (ng/μl)	*M (ng/g soil)
1	0	0	0	0	0	0.105316	0.07955	0.02646	0.070442	28.1768	0	0	0.00017	0.000056	0.0224
2	0.113935	0.034209	0.034209	0.060784	24.3136	0	0	0	0	0	0	0	0	0	0
3	0.59176	0.01329	0.94568	0.51691	206.764	0.00934	0.01444	0	0.007927	3.1708	0	0	0	0	0
4	0.01176	0.098815	0.36945	0.160008	64.0032	0.013363	0.00578	0	0.006381	2.5524	0.000079	0.00007	0	0.000049	0.0196
*M					73.7702					8.475					0.0105

*M: Mean

Table A6 Quantification of *C. beticola* DNA in Lower Saxonian field 1

No.	Layer1					Layer2					Layer3				
	R1	R2	R3	*M (ng/μl)	*M (ng/g soil)	R1	R2	R3	*M (ng/μl)	*M (ng/g soil)	R1	R2	R3	*M (ng/μl)	*M (ng/g soil)
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4	0.004070	0.004070	0.033480	0.013873	5.5492	0.003341	0.014215	0.014215	0.01059	4.236		0.0241227	0	0.008041	3.2164
5	0.0019772	0.00799	0	0.003322	1.3288	0	0	0	0	0	0	0.00878	0	0.002927	1.1708
6	0.06761	0	0	0.022537	9.0148	0.02120	0.06959	0.02120	0.03733	14.932	0	0.004248	0	0.001416	0.5664
7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
9	0	0.00527	0	0.001757	0.7028	0	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
*M					1.65956					1.9168					0.49536

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Table A7 Quantification of *C. beticola* DNA in Lower Saxonian field 2

No.	Layer1					Layer2					Layer3				
	R1	R2	R3	*M (ng/μl)	*M (ng/g soil)	R1	R2	R3	*M (ng/μl)	*M (ng/g soil)	R1	R2	R3	*M (ng/μl)	*M (ng/g soil)
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	0.0118	0	0.0118	0.007867	3.1468	0.004214	0	0	0.001405	0.562	0.0036144	0	0	0.001205	0.482
3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0.00409	0	0	0.001363	0.5452	0.010060	0	0	0.003353	1.3412
9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
*M					0.31468					0.11072					0.18232

*M: Mean

Table A8 Quantification of *C. beticola* DNA in Lower Saxonian field 3

No.	Layer1					Layer2					Layer3				
	R1	R2	R3	*M (ng/μl)	*M (ng/g soil)	R1	R2	R3	*M (ng/μl)	*M (ng/g soil)	R1	R2	R3	*M (ng/μl)	*M (ng/g soil)
1	0	0.0015539	0	0.000518	0.2072	0.09256	0.3304	0.60024	0.341067	136.4268	0.106754	0.35105	0.35105	0.269618	107.8472
2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5	0.01477	0.07441	0.106831	0.065337	26.1348	0.001002	0.607006	0.607006	0.405005	162.002	0	0	0	0	0
6	0.00223	0.0105825	0.0105825	0.007798	3.1192	2.83556	0.469533	0.469533	1.258209	503.2836	0.131609	0.0857355	0.0857355	0.101027	40.4108
7	0.00186	0	0	0.00062	0.248	0	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
*M					2.97092					80.17124					14.8258

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Table A9 Quantification of *C. beticola* DNA in Lower Saxonian field 4

No.	Layer1					Layer2					Layer3				
	R1	R2	R3	*M (ng/μl)	*M (ng/g soil)	R1	R2	R3	*M (ng/μl)	*M (ng/g soil)	R1	R2	R3	*M (ng/μl)	*M (ng/g soil)
1	0.02999	0.002024	0.002024	0.01136	4.544	0	0	0.02992	0.0099	3.96	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0.0054352	0.002021	0.002021	0.0031	1.24	0.029396	0.029396	0.006695	0.0218	8.72
6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0.007	0	0.0023	0.92	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
*M					0.4544					0.612					0.872

*M: Mean

Table A10 Quantification of *C. beticola* DNA in Lower Saxonian field 5

No.	Layer1					Layer2					Layer3				
	R1	R2	R3	*M (ng/μl)	*M (ng/g soil)	R1	R2	R3	*M (ng/μl)	*M (ng/g soil)	R1	R2	R3	*M (ng/μl)	*M (ng/g soil)
1	0.39333	0.75267	0.75267	0.63289	253.156	0.0081	0	0	0.0027	1.08	0.0016209	0.0016209	0.0016209	0.001621	0.6484
2	0.91497	0.141002	0.91497	0.656981	262.7924	0.07479	0.01556	0.07479	0.055047	22.0188	0.0036	0.0075545	0.0075545	0.006236	2.4944
3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4	0	0	0.015843	0.005281	2.1124	0.00172	0	0	0.000573	0.2292	2.1714	0.20296	2.1714	1.515253	606.1012
5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
9	0.09949	0.09949	0.018241	0.072407	28.9628	0	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
*M					54.70236					2.3328					60.9244

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Table A11 Quantification of *C. beticola* DNA in Lower Saxonian field 6

No.	Layer1					Layer2					Layer3				
	R1	R2	R3	*M (ng/μl)	*M (ng/g soil)	R1	R2	R3	*M (ng/μl)	*M (ng/g soil)	R1	R2	R3	*M (ng/μl)	*M (ng/g soil)
1	0.002540	0.0483	0.0483	0.033047	13.2188	0.0086	0.008	0.008	0.0082	3.28	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6	0.02635	0.010674	0.010674	0.015899	6.3596	0.0411	0.0411	0.0411	0.0411	16.44	0.00260	0.067240	0.00260	0.024147	9.6588
7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
9	0	0	0	0	0	0	0	0	0	0	0.00141	0	0	0	0
10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
*M					1.95784					1.972					0.96588

*M: Mean

Table A12 Quantification of *C. beticola* DNA in USA field 1

No.	Layer1					Layer2					Layer3				
	R1	R2	R3	*M (ng/μl)	*M (ng/g soil)	R1	R2	R3	*M (ng/μl)	*M (ng/g soil)	R1	R2	R3	*M (ng/μl)	*M (ng/g soil)
1	0.204088	0.20364	0.24759	0.218439	87.37573	0.02299	0.023904	0.03641	0.027768	11.1072	0	0	0	0	0

Table A13 Quantification of *C. beticola* DNA in USA field 2

No.	Layer1					Layer2					Layer3				
	R1	R2	R3	*M (ng/μl)	*M (ng/g soil)	R1	R2	R3	*M (ng/μl)	*M (ng/g soil)	R1	R2	R3	*M (ng/μl)	*M (ng/g soil)
1	0.00194478	0.00315	0	0.00169826	0.679304	0.00144	0	0	0.00048	0.192	0	0	0	0	0

Table A14 Determination of *C. beticola* DNA degradation in climate chamber

Month	Dilution 1					Dilution 2				
	R1	R2	R3	*M (ng/μl)	*M (ng/g soil)	R1	R2	R3	*M (ng/μl)	*M (ng/g soil)
1	2.03639	1.55	2.27121	1.95253333	781.0133	0.026724	0.03336	0.0182532	0.0261124	10.44496
2	0.080904	0.114466	0.125755	0.10704167	42.81667	0.0024939	0.0029300	0.0024939	0.00263927	1.055708
3	0.088290	0.0924044	0.0924044	0.09103293	36.41317	0.0015171	0.002369	0.0024939	0.00212667	0.850668
4	0.016621	0.0187514	0.024295	0.01988913	7.955652	0.0019965	0.0019965	0.0019965	0.0019965	0.7986
5	0.0041687	0.0064133	0.010144	0.00690867	2.763468	0.0017785	0.0017785	0.0017785	0.0017785	0.7114
6	0.0068566	0.005844	0.005844	0.00618153	2.472612	0.00177	0.00177	0.00177	0.00177	0.708

Table A15 Quantification of *C. beticola* antigen in Bavarian field 1

No.	Layer 1		Layer 2		Layer 3	
	**OD Values	µg mycelium/g soil	**OD Values	µg mycelium/g soil	**OD Values	µg mycelium/g soil
1	0.12	151	0.14	184.3333	0.14	184.3333
2	0.12	151	0.14	184.3333	0.15	201
3	0.12	151	0.14	184.3333	0.15	201
4	0.12	151	0.14	184.3333	0.15	201
5	0.12	151	0.14	184.3333	0.15	201
6	0.12	151	0.14	184.3333	0.15	201
7	0.12	151	0.14	184.3333	0.15	201
8	0.12	151	0.14	184.3333	0.15	201
9	0.12	151	0.14	184.3333	0.15	201
10	0.12	151	0.14	184.3333	0.15	201
*M		151		184,3333		199.3333

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Table A16 Quantification of *C. beticola* antigen in Bavarian field 2

No.	Layer 1		Layer 2		Layer 3	
	**OD Values	µg mycelium/g soil	**OD Values	µg mycelium/g soil	**OD Values	µg mycelium/g soil
1	0.20	284.3333	0.16	217.6667	0.19	267.6667
2	0.20	284.3333	0.16	217.6667	0.19	267.6667
3	0.20	284.3333	0.16	217.6667	0.19	267.6667
4	0.20	284.3333	0.16	217.6667	0.19	267.6667
5	0.20	284.3333	0.16	217.6667	0.19	267.6667
6	0.20	284.3333	0.16	217.6667	0.19	267.6667
7	0.20	284.3333	0.16	217.6667	0.19	267.6667
8	0.20	284.3333	0.16	217.6667	0.19	267.6667
9	0.20	284.3333	0.16	217.6667	0.19	267.6667
10	0.20	284.3333	0.16	217.6667	0.19	267.6667
*M		284.3333		217.6667		267.6667

*M: Mean

**OD Values: Optical density of the antigen in sample.

Table A17 Quantification of *C. beticola* antigen in Bavarian field 3

No.	Layer 1		Layer 2		Layer 3	
	**OD Values	µg mycelium/g soil	**OD Values	µg mycelium/g soil	**OD Values	µg mycelium/g soil
1	0.13	167.6667	0.13	167.6667	0.12	151
2	0.13	167.6667	0.13	167.6667	0.13	167.6667
3	0.12	151	0.12	167.6667	0.12	151
4	0.13	167.6667	0.13	167.6667	0.13	167.6667
5	0.13	167.6667	0.13	167.6667	0.12	151
6	0.12	151	0.13	167.6667	0.12	151
7	0.13	167.6667	0.13	167.6667	0.12	151
8	0.13	167.6667	0.13	167.6667	0.12	151
9	0.13	167.6667	0.13	167.6667	0.12	151
10	0.13	167.6667	0.13	167.6667	0.12	151
*M		164.3334		167.6667		154.3333

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Table A18 Quantification of *C. beticola* antigen in Bavarian field 4

No.	Layer 1		Layer 2		Layer 3	
	**OD Values	µg mycelium/g soil	**OD Values	µg mycelium/g soil	**OD Values	µg mycelium/g soil
1	0.17	234.3333	0.23	334.3333	0.28	417.6667
2	0.18	251	0.20	284.3333	0.18	251
3	0.19	267.6667	0.19	267.6667	0.23	334.3333
4	0.18	251	0.20	284.3333	0.24	351
5	0.18	251	0.20	284.3333	0.18	251
6	0.18	251	0.19	267.6667	0.20	284.3333
7	0.18	251	0.20	284.3333	0.20	284.3333
8	0.18	251	0.20	284.3333	0.23	334.3333
9	0.18	251	0.19	267.6667	0.24	351
10	0.19	267.6667	0.20	284.3333	0.20	284.3333
*M		252.6667		284.3333		314.3333

*M: Mean

**OD Values: Optical density of the antigen in sample.

Table A19 Quantification of *C. beticola* antigen in Bavarian field 4. 1 year after the first sampling date

No.	Layer 1		Layer 2		Layer 3	
	**OD Values	µg mycelium/g soil	**OD Values	µg mycelium/g soil	**OD Values	µg mycelium/g soil
1	0.15	201	0.13	167.666	0.12	151
2	0.15	201	0.13	167.666	0.12	151
3	0.15	201	0.13	167.666	0.12	151
4	0.15	201	0.13	167.666	0.12	151
*M		201		167.666		151

Table A20 Quantification of *C. beticola* antigen in Lower Saxonian field 1

No.	Layer 1		Layer 2		Layer 3	
	**OD Values	µg mycelium/g soil	**OD Values	µg mycelium/g soil	**OD Values	µg mycelium/g soil
1	0.23	334.3333	0.23	334.3333	0.25	367.6667
2	0.23	334.3333	0.23	334.3333	0.25	367.6667
3	0.24	351	0.22	317.6667	0.25	367.6667
4	0.22	317.6667	0.22	317.6667	0.25	367.6667
5	0.23	334.3333	0.23	334.3333	0.25	367.6667
6	0.26	384.3333	0.22	317.6667	0.25	367.6667
7	0.22	317.6667	0.22	317.6667	0.25	367.6667
8	0.25	367.6667	0.22	317.6667	0.25	367.6667
9	0.22	317.6667	0.22	317.6667	0.25	367.6667
10	0.22	317.6667	0.22	317.6667	0.25	367.6667
*M		337.6667		322.6667		367.6667

*M: Mean

**OD Values: Optical density of the antigen in sample.

Table A21 Quantification of *C. beticola* antigen in Lower Saxonian field 2

No.	Layer 1		Layer 2		Layer 3	
	**OD Values	µg mycelium/g soil	**OD Values	µg mycelium/g soil	**OD Values	µg mycelium/g soil
1	0.28	417.6667	0.26	384.3333	0.26	384.3333
2	0.31	467.6667	0.29	434.3333	0.27	401
3	0.31	467.6667	0.28	417.6667	0.26	384.3333
4	0.28	417.6667	0.26	384.3333	0.26	384.3333
5	0.31	467.6667	0.29	434.3333	0.28	417.6667
6	0.31	467.6667	0.28	417.6667	0.27	401
7	0.31	467.6667	0.26	384.3333	0.26	384.3333
8	0.31	467.6667	0.28	417.6667	0.26	384.3333
9	0.31	467.6667	0.29	434.3333	0.27	401
10	0.31	467.6667	0.26	384.3333	0.26	384.3333
*M		457.6667		409.3333		392.6667

Table A22 Quantification of *C. beticola* antigen in Lower Saxonian field 3

No.	Layer 1		Layer 2		Layer 3	
	**OD Values	µg mycelium/g soil	**OD Values	µg mycelium/g soil	**OD Values	µg mycelium/g soil
1	0.36	551	0.33	501	0.30	451
2	0.37	567.6667	0.32	484.3333	0.30	451
3	0.35	534.3333	0.32	484.3333	0.30	451
4	0.33	501	0.32	484.3333	0.30	451
5	0.35	534.3333	0.33	501	0.30	451
6	0.34	517.6667	0.32	484.3333	0.30	451
7	0.35	534.3333	0.33	501	0.30	451
8	0.36	551	0.32	484.3333	0.30	451
9	0.36	551	0.33	501	0.30	451
10	0.36	551	0.32	484.3333	0.30	451
*M		539.3333		491		451

*M: Mean

**OD Values: Optical density of the antigen in sample.

Table A23 Quantification of *C. beticola* antigen in Lower Saxonian field 4

No.	Layer 1		Layer 2		Layer 3	
	**OD Values	µg mycelium/g soil	**OD Values	µg mycelium/g soil	**OD Values	µg mycelium/g soil
1	0.23	334.3333	0.23	334.3333	0.25	367.6667
2	0.22	317.6667	0.22	317.6667	0.25	367.6667
3	0.24	351	0.24	351	0.25	367.6667
4	0.23	334.3333	0.23	334.3333	0.25	367.6667
5	0.22	317.6667	0.22	317.6667	0.25	367.6667
6	0.23	334.3333	0.22	317.6667	0.25	367.6667
7	0.23	334.3333	0.22	317.6667	0.25	367.6667
8	0.23	334.3333	0.22	317.6667	0.25	367.6667
9	0.23	334.3333	0.22	317.6667	0.25	367.6667
10	0.23	334.3333	0.22	317.6667	0.25	367.6667
*M		332.6667		324.3334		367.6667

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Table A24 Quantification of *C. beticola* antigen in Lower Saxonian field 5

No.	Layer 1		Layer 2		Layer 3	
	**OD Values	µg mycelium/g soil	**OD Values	µg mycelium/g soil	**OD Values	µg mycelium/g soil
1	0.35	534.3333	0.28	417.6667	0.27	401
2	0.35	534.3333	0.28	417.6667	0.24	351
3	0.35	534.3333	0.31	467.6667	0.24	351
4	0.34	517.6667	0.28	417.6667	0.24	351
5	0.35	534.3333	0.28	417.6667	0.24	351
6	0.33	501	0.31	467.6667	0.24	351
7	0.34	517.6667	0.28	417.6667	0.24	351
8	0.35	534.3333	0.31	467.6667	0.24	351
9	0.34	517.6667	0.28	417.6667	0.24	351
10	0.35	534.3333	0.28	417.6667	0.24	351
*M		526		432.6667		356

*M: Mean

**OD Values: Optical density of the antigen in sample.

Table A25 Quantification of *C. beticola* antigen in Lower Saxonian field 6

No.	Layer 1		Layer 2		Layer 3	
	**OD Values	µg mycelium/g soil	**OD Values	µg mycelium/g soil	**OD Values	µg mycelium/g soil
1	0.15	201	0.17	234.3333	0.17	234.3333
2	0.15	201	0.17	234.3333	0.17	234.3333
3	0.13	167.6667	0.17	234.3333	0.17	234.3333
4	0.14	184.3333	0.18	251	0.17	234.3333
5	0.14	184.3333	0.17	234.3333	0.17	234.3333
6	0.14	184.3333	0.16	217.6667	0.17	234.3333
7	0.15	201	0.17	234.3333	0.17	234.3333
8	0.15	201	0.16	217.6667	0.17	234.3333
9	0.15	201	0.17	234.3333	0.17	234.3333
10	0.15	201	0.17	234.3333	0.17	234.3333
*M		192.6667		232.6667		234.3333

*M: Mean

**OD Values: Optical density of the antigen in sample.

Table A26 Quantification of *C. beticola* antigen in USA field 1

No.	Layer 1		Layer 2		Layer 3	
	**OD Values	µg mycelium/g soil	**OD Values	µg mycelium/g soil	**OD Values	µg mycelium/g soil
1	0.16	217.6667	0.16	217.6667	0.15	201

**OD Values: Optical density of the antigen in sample.

Table A27 Quantification of *C. beticola* antigen in USA field 2

No.	Layer 1		Layer 2		Layer 3	
	**OD Values	µg mycelium/g soil	**OD Values	µg mycelium/g soil	**OD Values	µg mycelium/g soil
1	0.15	201	0.15	201	0.15	201

**OD Values: Optical density of the antigen in sample.

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Table A28 Determination of *C. beticola* antigen degradation in climate chamber

Month	Dilution 1		Dilution 2	
	**OD Values	µg mycelium/g soil	**OD Values	µg mycelium/g soil
1	0.31	467.66	0.27	401
2	0.31	467.66	0.27	401
3	0.31	467.66	0.27	401
4	0.3	451	0.26	384.33
5	0.26	384.33	0.23	334.33
6	0.21	301	0.2	284.33

**OD Values: Optical density of the antigen in sample.

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