A INTRODUCTION

Sooty blotch disease of apple may occur worldwide in warm and humid climates and is a plant disease, which can cause considerable crop loss by affecting fruit quality and yield (BATZER et al. 2002; WILLIAMSON and SUTTON 2000). This disease is caused by a group of fungal pathogens that are colonising the cuticle of fruit (JOHNSON 1996). Indeed, these fungi are epiphytes, which don't penetrate the epidermis of the fruit. However, they may cause brownish-black, washy spots of different size on the fruit surface and in case of strong infestation can cover the whole fruit. Normally, such fruit are not suited as dessert fruit. Hence, control of this disease is extremely difficult in some regions, since despite of repeated fungicide applications insufficient results are achieved. In many cases sooty blotch is suppressed by fungicides, which are routinely used in scab control, *Venturia inaequalis* f. sp. *mali* R. Menon. However, there is a trend of planting scab resistant apple cultivars such as `Topaz`, in order to reduce the number of fungicide treatments against scab of apple. As a consequence of these reduced fungicide applications the risk potential of sooty blotch incidence is increasing and even may cause problems in such regions, where this disease has been sufficiently controlled so far. Also, recent reports indicate a higher sensitivity of ´Topaz´ towards sooty blotch (observations of different growers from the Rhineland, Germany).

In the past numerous studies have been devoted to the phenomenon of sooty blotch. Originally, it was assumed that only one fungus, namely *Gloeodes pomigena* (Schwein.) Colby, was causing this disease (WILLIAMSON and SUTTON 2000). Nowadays it is known that a pathogen complex of up to 20 different fungal species are evoking sooty blotch. Hence, enormous difficulties are hampering the clarification of this disease. The composition of the pathogen spectrum can vary from year to year due a number of different factors, such as temperature, moisture, rainfall, wind, solar irradiation, overwintering hosts or others. When overcoming the difficulties of isolation of the fungi, exceptional slow growth of the fungal cultures *in vitro* is encountered. In some cases it may take up to 3 months until a fungal colony has reached a diameter of a few centimetres. However, fungal cultures often can not be determined due to lacking conidia formation *in vitro*.

In the present study causal organisms of sooty blotch disease, isolated from apple fruit in different regions of Germany (Lake Constance region to Rhineland), were to be characterised by classical mycological methods. For this purpose, both macroscopic and microscopic photos of the respective fungal culture *in vitro* as well as of fungal characters were taken. In addition, the most important characters such as hyphae length or conidia width were measured. For a precise assignment and support, the results of the classical mycological examinations were verified through DNA analytical tests by sequencing rDNA LSU and rDNA ITS 1+2. After identification and classification of selected sooty blotch causing fungi from Germany isolates were analysed for mycotoxins. Due to reports of VENKATASUBBAIAH et al. (1995) these fungi may release mycotoxins of the potentially harmful trichothecene group. Therefore, fungal isolates and apple products were analysed employing HPLC/MS/MS in order to elucidate the potential risk to apple consumers in Germany. Furthermore, it may be speculated that stressors, such as temperature extremes, sublethal fungicide applications and variation in supply with nutrients may facilitate mycotoxin production. This question was

answered employing the model fungus *Trichothecium roseum* (Pers.) Link and the mycotoxins trichothecin and trichothecolone.

Mycotoxins

Investigations by VENKATASUBBAIAH et al. (1995) point out that at least *Peltaster fructicola* Johnson, T. B. Sutton & Hodges might be a potential mycotoxin producer, since the fungus is able to synthesise the mycotoxins trichothecolone and trichothecolone acetate belonging to the group of the type B-trichothecenes. Hence, the two aforementioned, in liquid culture produced mycotoxins are toxicologically precarious (SMITH et al. 1985; DEUTSCHE GES. FÜR ERNÄHRUNG 1996). It is to be considered that in practice except for these two substances, which were specified before, additional trichothecenes are being formed on the fruit. So far, about 170 trichothecenes are well-known, which are produced by different fungi (SCHÜTTE 2000). It still is unclear, whether the pathogens of the sooty blotch disease are able to form additional mycotoxins. Depending on their biosynthetic pathway trichothecenes are subdivided into four different groups, assigned as type A-, B-, C- and D-trichothecenes. Their common structure consists of a three ring system with an additional epoxy group at C(12). These trichothecenes may cause a whole spectrum of different toxic effects both in humans and in animals. If the toxins are taken up with the food, among others the digestive tract, the nervous system, the blood formation and the marrow are negatively affected. By suppression of the protein synthesis and reciprocal effect in the cell membranes an impairment of the immune system is induced (PESTKA et al. 1994). Due to the attenuation of the immune system the body-own defence diminishes, and secondary infections by bacteria and viruses arise. Numerous cases of death are due to these secondary infections. Pigs and other monogastrics, including human beings, respond most sensitively to trichothecenes. First reactions are food refusal and indisposition. In the further process vomiting, diarrhoea and apathy may occur (PRELUSKY et al. 1994). Since apples belong to the group of the most frequently consumed fruit one of the objectives of this project was to elucidate the potential risk of contaminated products for consumers and authorities. Therefore liquid cultures of the sooty blotch disease pathogens, as well as apples with symptoms of the sooty blotch disease and apple products were investigated for the occurrence of the mycotoxins trichothecin, trichothecolone, nivalenol, deoxynivalenol, T-2 toxin and diacetoxyscirpenol. It was to be clarified, whether the German sooty blotch fungi are able to produce the mycotoxins specified before both in liquid culture and *in vivo* on apple fruit surface. Furthermore, an answer should be given to the question, whether a diffusion of the toxins through the cuticle and epidermis into the pulp is taking place in infested apples.

Stressors

Contamination of food with fungal toxins represents a major toxicological concern and a continuous threat to the health of both animals and humans (ROTTER et al. 1996; JOFFE 1986; UENO 1983). Among these toxins trichothecenes are regarded as one of the most important mycotoxins in the world. Minimization of the problem can be achieved for example by effective control of the toxin producing fungi. There are a number of appropriate fungicides for the various plant/pathogen systems. In cereal crops/*Fusarium spec.* pyrimidine, triazole and imidazole fungicides are frequently used (MATTHIES et al. 1999). However, in some cases in *Fusarium* headblight even increases of trichothecin level were observed despite of

fungicide application, though disease severity was effectively reduced (LIENEMANN et al. 2002; MATTHIES et al. 1999). *In vitro* studies employing *Fusarium graminearum* and selected fungicides, e.g. thiobendazole and tebuconazole, clearly documented that at sublethal concentrations of these compounds release of 3-acetyldeoxynivalenol (3-ADON), which is one of the most frequently occurring mycotoxins in cereal production, was increased relative to mycelial growth. This may be regarded as one of the potential reasons for the obvious stimulation of trichothecin production, as also hypothesized in earlier studies of GAREIS and CEYNOWA (1994).

If this phenomenon is also occurring in other plant/mycotoxin producing pathogen systems, is unknown so far. Besides of cereals, also horticultural plants and fruits are potentially endangered by mycotoxin releasing fungi, e.g. trichothecin in grapes (SCHWENK et al. 1989), patulin in apple juice (WATANABE and SHIMIZU 2005), fumonisins in asparagus and garlic (SEEFELDER et al. 2004) contamination of almond, pistachio and walnut by aflatoxins (CAMPBELL et al. 2003); in this case, use of appropriate fungicides is also an effective way of control. The objective of the present study therefore was to elucidate whether sublethal fungicide doses, temperature effects as well as the supply of different nutrients from deficiency to overspill stimulate mycotoxin production of the pathogen. For testing this, *Trichothecium roseum* (Pers.) Link has been selected as model fungus, since it is an ubiquist and found world-wide in numerous horticultural crops, e.g. grape, apple, pear, Citrus, bean, coffee ... (DOMSCH et al. 1993). In addition, besides other metabolites *T. roseum* is capable of producing the mycotoxins trichothecin and trichothecolone both belonging to the group of the type B-trichothecenes (DOMSCH et al. 1993; SCHWENK et al. 1987). For control of the fungus, two mesostemic (kresoxim-methyl and trifloxystrobin), a protective one (fenhexamid) and a combination product integrating both protective and systemic properties (fludioxonil and cyprodinil) were selected. This was to be shown as a first approach *in vitro*. The present study was based on the hypothesis that mycotoxin production is enhanced at sublethal fungicide doses in *T. roseum.*

The questions to be answered in this study are:

1. What are the mycological and morphological characters of the pathogens of the sooty blotch disease?

2. What is the classification of the individual, in Germany isolated pathogens of the sooty blotch disease, based on DNA sequence analyses?

3. Are pathogens of the sooty blotch disease, isolated in Germany able to form mycotoxins belonging to the group of trichothecenes?

4. To what extent are stressors influencing the ability of mycotoxin formation of a fruit colonising fungal organism, as documented in *Trichothecium roseum*?

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B Biological characterisation of sooty blotch fungi in apple (*Malus* x *domestica* Borkh.) of Central Europe

1. INTRODUCTION

1.1 Significance of sooty blotch in apple

Sooty blotch disease of apple is caused by a certain group of cuticle inhabiting fungi. The disease is frequently occurring in humid and temperate climate zones of the world (WILLIAMSON and SUTTON 2000). In Germany, the disease appears most frequently in humid warm fruit growing areas like the Lake Constance region or in the Rhineland. But there is reference that this disease can be found in all regions, where apples are grown (NOGA et al. 2001). Neither growth nor fruit development is influenced by these fungi. However the pathogens may have a negative impact on the outer fruit quality by producing brownish-black, washy spots of different size on the fruit surface, especially in apple cultivars with a light peel. These fungi are exclusively colonizing the cuticle without penetrating it (JOHNSON 1996). Apples with symptoms of sooty blotch are generally refused by the consumer and excluded from sales according to EU quality standards. This may result in economic losses up to 90 % (BATZER et al. 2002, NOGA et al. 2001, WILLIAMSON and SUTTON 2000). The pathogens of sooty blotch disease may be controlled by fungicide applications exerted from the time immediately after bloom to shortly before harvest. It was even shown that under very humid and warm growing conditions the disease could not be sufficiently controlled despite of intensive plant protection programs (HARTMAN 1995). In order to control pathogens successfully, it is important to be endued with precise knowledge about the taxonomy and ecology of the fungi. Working with this topic is complicated, since isolation of the pathogens of the apple peel proved to be difficult. In some cases conidia that are essential for identification of the respective fungus were missing. Also growth of the cultures *in vitro* is very slow. Therefore, some of the fungi were not characterised yet. In five different growing areas of Germany apples of the cultivars ‚Sir Prize', ‚Golden Delicious', ‚Pilot' and others, which showed symptoms of sooty blotch disease, were collected and examined in the time from 1998 to 2000. Basis for the own studies for the biological characterisation of these phytopathogenic fungi is a collection of more than 50 different isolates. The objective of the present study is to characterise sooty blotch fungi isolated in Germany and being representative for Central Europe*.

1.2 State of knowledge on pathogens causing sooty blotch disease in apple (*Malus* x *domestica* Borkh.)

Symptoms of sooty blotch

Sooty blotch disease of apple (*Malus x domestica* Borkh.) can be recognised as brownish-black, washy spots of different size on the fruit surface, which can only insufficiently be removed by hard mechanical rubbing (Fig. 1). Spreading of conidia on the fruit surface by down-dripping rain droplets leads to narrow, lace-shaped (tongue-shaped) bands extending

* momentarily other groups are working on American and Asian sooty blotch fungi (communication M. Gleason)

from the stem cavity to the calyx end. At strong infestation the fruit surface can be almost completely covered with sooty coloured blackish coating. The colonies vary in colour, shape and size depending on the respective fungi that are present on the apple cuticle. They consist of a mycelial network that in some cases is able to produce fruiting bodies in form of pycnothyria. The individual fungal species differ both in number, size and arrangement of the fruiting bodies, as well as in the type of mycelial growth. The colonies vary from olive-green to black and can be small and circular, or big and diffuse. As a consequence of secondary spread via conidia some colonies cover a great part of the fruit surface, whereas other colonies remain individual and do not spread out. If the symptoms are appearing together with the flyspeck disease, *Schizothyrium pomi* (Mont. & Fr.) Arx, anamorph: *Zygophiala jamaicensis* E.W. Mason, the disease pattern is called SBFS (*German*: Regenfleckenkrankheit) (NOGA et al. 2001; WILLIAMSON and SUTTON 2000; JOHNSON et al. 1997; GROVES 1933).

Fig. 1: apple fruit contaminated with colonies of sooty blotch fungi

Hosts

In the past, several researchers believed to have isolated the disputed species "*Gloeodes pomigena*" from *Malus* x *domestica* Borkh. and many different plants, which they believed to be the reservoir or overwintering hosts (BAINES 1932; COLBY 1920; GARDNER 1931; HICKEY 1960; ZARING 1929). They thought they were dealing with a clearly defined fungal organism. The formerly used denomination is to be challenged (cp. JOHNSON et al. 1997; cp. CANNON 1996 see chap. C 1.1). Therefore, the observations mentioned in the literature of the years 1920 to 1960 are only to be assessed as preliminary only and as possibly not correct. In 2005, MAYR and SPÄTH observed a significant correlation between infected *Rubus spp.* and number of spots on apple. Whenever he attached twigs of *Rubus spp.* to the canopy of apple trees the infection of fruit with sooty blotch increased. If there was no *Rubus spp.*, there was no infection with sooty blotch. It was in 2003/2004, when TRAPMAN (2005) applied high amounts of fungicides during wintertime and spring to apple trees in order to test, whether the sooty blotch fungi overwinter in the apple trees or not. He found out that in spite of the lethal fungicidal doses fruits were nevertheless infected with sooty blotch fungi during the following summer season.

Disease development and epidemiology (cp. also chap. C 1.1)
Due to the aforementioned observations it is currently accepted that the fungi or at least some of the fungi of the pathogen complex of sooty blotch disease are growing from late spring to autumn in apple trees and change their host in autumn to overwinter on *Rubus spp.* e. g. blackberry or other still unknown hosts until late spring or early summer before infecting developing fruit on apple trees again. Some researchers assume that conidia, in case they are formed, are spread by wind and rain. But details of the life cycle of sooty blotch fungi are still unknown (MAYR and SPÄTH 2005; TRAPMAN 2005; WILLIAMSON et al. 2000; JONES 1996).

1.3 Sooty blotch and sooty moulds in other fruit species

The symptoms of sooty blotch disease on plant surfaces were described for different fruit and vegetable cultivars worldwide. In humid regions sooty blotch disease causes great damage to the appearance of citrus fruit by superficial fungal colonies that cover the peel. The causal fungus is able to cause cracking of the cuticle, but doesn't invade the epidermis. The sooty blotches consist of branched, reticular, sometimes thick-walled brown to black hyphae (MENGE 1993). Examinations of BITANCOURT (1934) on sooty blotch fungi on sweet orange (*Citrus sinensis* Osbeck) in Brazil resulted in the conclusion that other fungi than those of the sooty blotch disease complex of apple were found. BITANCOURT described a variety with larger thyriothecia, *Stomiopeltis citri* var. *citri* and with smaller thyriothecia *Stomiopeltis citri* var. *minor* n. var. pathogens. The thyriothecia or shield-shaped perithecia show a meandric-plectenchymatic structure. The asci are located in a paraphysoid tissue and contain bicellular, hyaline spores. For this pathogen BITANCOURT suggested the name *Stomiopeltis citri* n. sp.

On Mango (*Mangifera spec.*) fruit surface, symptoms of „tear-stain" were discovered that are caused by *Dothiorella mangiferae* Syd. & P. Syd. Similar symptoms can also be induced by *Dothiorella dominicana* Petr. & Cif. and *Colletotrichum gloeosporioides* Penz. They belong to the same spectrum of pathogens that cause stem-end-rot of mango. The putative teleomorph of *Dothiorella mangiferae* is *Botryosphaeria parva* Pennycock & Samuels that was described for kiwi fruit (*Actinidia spec*.) (PLOETZ et al. 1994).

Chaetothyrina musarum (Speg.) Theissen is the causal organism of sooty blotch of bananas. As host of these fungi *Musa spec.*, *Musa paradisiaca* L. (Musaceae), *Smilax spp.* (Liliaceae) and numerous other plant species in tropical America and Africa are described. *C. musarum* forms itself a superficially spreading mycelium on stacks, leafs and fruits. It consists of hyaline or more often brownish hyphae with dark setae that are growing on the cuticle. *Chaetothyrina* can be diferentiated from the sooty blotch fungus *Stomiopeltis* by the fruiting bodies covered with setae (THEISSEN 1914).

In tropic climates besides sooty blotch fungi also sooty mould fungi, forming black, membranous coating on plant surfaces, are being found frequently. Sooty mould fungi belong to the Dothideales and Chaetothyriales, and often sooty blotch and sooty mould fungi are able to grow with one another or one above the other, without limiting each other (LIM 1975; MUELLER et al. 2004).

2. MATERIAL AND METHODS

2.1 Fungal culture collection

2.1.1 Designation of the cultures

The listed fungal isolates are components of a fungal culture collection, which was established in the period between 1996 and 2001. The fungi of this culture collection were isolated from apple fruit showing symptoms of sooty blotch disease from orchards in the Lake Constance area and in the Rhineland (Germany). Subsequently they were taken in culture.

2.1.2 Preparation of culture media

The culture media were autoclaved at 121° C and 1 bar for 20 min. The pH-value of all media was adjusted to 5.6.

Malt extract agar (MEA)
48 g MEA Merck (Darmstadt)
1000 ml a. demin.

Potato Dextrose Agar (PDA)
39 g PDA Merck (Darmstadt)
1000 ml a. demin.

2.1.3 Cultivation of the fungi

The fungi were cultivated on artificial culture media (MEA and PDA) in the dark. For the purpose of better and faster growth the Petri dishes freshly inoculated with a mycelium-pellet (diameter 6 mm) were placed for 14/30 days at room temperature under exclusion of light and then returned to the culture collection. The stock cultures were stored in PDA-tubes at 6 °C.

2.2 Fungal cultures

2.2.1 Macroscopic documentation of fungal cultures

For evaluation of morphological differences *in vitro* the fungal cultures of the pathogens of the sooty blotch disease were photographed. It was made use of a Leica R 8 camera with a Leica macro lens R 2.8 / 60 (Solms, Germany) and a RSX 100 II film of Agfa (Leverkusen, Germany). The slides were digitalized with a Nikon film scanner Coolscan V ED (Düsseldorf, Germany).

2.2.2 Microscopic presentation of different organs of fungal cultures

The microscopic pictures were compiled in the light field- and DIC-mode with a magnification of 400x to 1250x with an Axioskop 50 microscope of Zeiss (Oberkochen / Göttingen, Germany). As required some objects were microscopied with an accordingly lower magnification. As digital camera a Zeiss AxioCam MRc5 in combination with the software AxioVision AC 4.2 was utilized. This software supplied a possibility of measurement. In