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Thomas Schröder

On the geographic variation of *Ciboria batschiana* (Zopf) Buchwald, the main pathogenic fungus on acorns of *Quercus robur* and *Q. petraea* in Europe

Abstract: Mycelial growth of the pathogenic fungus *Ciboria batschiana* (Zopf) Buchwald was examined at nine temperature levels between –3°C and +35°C using pure cultures of the fungus from eleven different provenances located in Germany, Poland, the Czech Republic and Slovenia. The fungus was isolated mainly from cotyledons of acorns of the oak species *Quercus robur* L. and *Quercus petraea* (Matt.) Liebl. Even at –3°C the mycelium of the fungus grew well. There were significant differences between the provenances in respect to growth rate and temperature tolerance, especially at low temperatures.

Additional key words: acorns, *Quercus*, seed storage, seed-borne fungi

Address: T. Schröder, Federal Biological Research Centre for Agriculture and Forestry (BBA), Institute for Plant Protection in Forests, Messeweg 11–12, D–38104 Braunschweig, Germany, t.schroeder@bbd.de

Introduction

The fungus *Ciboria batschiana* (Zopf) Buchwald (syn. *Sclerotinia pseudotuberosa* [Rehm] Rehm; anamorph: *Rhacodiella* sp.) belongs to the Ascomycetes (Discomycetes, Sclerotiniaceae) and is the most important pathogen of oak (*Quercus* spp.) acorns. At the time of acorn ripening the apothecia of the fungus develop on mummified cotyledons of the previous year´s acorns (Delatour 1984), which are present in litter. Masses of ascospores ejected from these apothecia infect ripe acorns when they fall to the ground.

Ciboria batschiana causes serious problems during acorn storage in Germany and other countries. The fungus is able to grow even at low temperatures, and when storage conditions are unfavourable, e.g. at storage temperatures slightly above freezing, whole seedlots can be destroyed during one winter's storage (Delatour et al. 1982). The microconidia of the *Rhacodiella* anamorphic state, which is often present on affected acorns during storage, are not capable of germination (Butin 1996), so the infection of healthy seeds during the storage period is caused by spreading mycelium of the fungus. The teleomorph develops on mummified acorns in the forest litter, in the following year at the earliest, so it is not a factor for the spread of infections to healthy acorns during artificial storage. At present, thermotherapy, a hot water bath of acorns at 41°C for two hours, is the only effective phytosanitary method for killing the fungus without reducing viablity of acorns (Delatour 1978; Delatour and Morelet 1979; Gille 1997).

Rehm characterized the fungus as a parasite as early as 1896, when it was regarded as very important due to an epidemic in Southern Germany (Franken) which destroyed acorn crops. While the infection of acorns by *Ciboria batschiana* has been posing storage problems in France (Morelet 1974), Germany and the Czech Republic (Urosevic 1959) for many decades, the fungus showed its pathogenic capacity in Poland only about seven years ago according to J. and B. Suszka (1997, 1999). B. Suszka (1999) reported that *Ciboria batschiana* was well known in Poland and Great Britain at the end of the 1970s but that the fungus

caused no losses during acorn storage. The same is reported from the Baltic States (Vasiliauskas 1996, personal communication). Hence during a colloquium of the "working group on acorn storage" in Braunschweig, Germany, the hypothesis was put forward that different pathogenic strains of *Ciboria batschiana* could exist in Europe. Starting in France, these would have then spread north- and eastwards to Poland (Wulf and Schröder 1997).

Though it can be assumed that in Germany the fungus *Ciboria batschiana* is present in every oak stand, the rate of infection differs depending on the geographic location. Investigations on methods for treatment and storage of acorns (Schröder 1999) revealed that strains from different locations in Germany differ in mycelial growth rate and culture morphology.

Aim of this investigation was to clarify if there are significant differences in mycelium growth and temperature tolerance of *Ciboria batschiana* strains of different European provenances. Therefore tests on the growth rate were carried out at nine different temperatures using pure cultures of the fungus *Ciboria batschiana* from eleven different geographic origins.

Methods

Eight strains of the fungus *Ciboria batschiana* were isolated from freshly collected acorns of the oak species *Quercus robur* L. and *Quercus petraea* (Matt.) Liebl. in the autumn of 1999. For comparison additional strains isolated from *Quercus petraea* (collection 1996), *Quercus rubra* L. (collection 1989) as well as from *Castanea sativa* Mill. (collection 1999) were included in the investigation (Fig. 1).

The whole pericarp was removed and acorns with an obvious infection by *Ciboria batschiana* (dark-brown to black cotyledons or typical sharp edged orange-brown points of infection on the surface of the cotyledons) were selected. The cotyledons of these acorns were cut into 4 mm thick discs and incubated on moist filter paper at room temperature. Outgrowing mycelium was transferred to 2% malt agar (MEA-) petri-dishes (diameter 90 mm, 20 ml MEA per dish). Subcultures were also plated on 2% MEA. The cultures were incubated at room temperature and diffuse daylight. From each provenance a single culture was used to produce all the subcultures for the different temperature levels of the investigation in order to avoid possible differences in growth rate caused by repeated subculturing. Inoculum pieces were extracted under sterile conditions using a cork borer (diameter 4 mm) and placed, with the mycelium side facing down, on the center of petri dishes containing 2% MEA. On the underside of the petri dish, two right-angled diameter lines for measuring the radial growth of the fungus were drawn, with the inoculum piece in the cross section. This method was used to avoid a subjective orientation of the persons marking the diameter growth.

Ten cultures per strain were incubated at the following temperatures: –3°C, 0°C, 5°C, 10°C, 15°C, 20°C, 25°C, 30°C and 35°C. Mycelium growth was measured before the mycelium reached the margin of the petri-dish. Each of the four marked lines was measured from the edge of the inoculum piece to the colony margin and the mean value of mycelial growth was calculated. Those cultures with no or very minute mycelium growth were transferred to a 20°C climate chamber at the end of the test to check whether the mycelium had remained viable at the test temperature or not.

For the statistical analysis the computer programs SigmaStat® 2.03 and SAS 6.12 were used. The Kolmogoroff-Smirnoff-Test was used to test the data for their GAUSS distribution and the F-Test for the analysis of variance. For the comparison of the mean values of the fungal strains the Student-Newman--Keuls-Test was used because it gives better results for large sample sizes and because of its better selectivity in comparison with the Tukey-Test, which could also have been applied (Lozán 1992).

Results

The mycelial growth of most strains of the fungus *Ciboria batschiana* was characterized by an increasing growth rate up to a temperature of 20°C. However, at a temperature of 25°C growth was greatly reduced (Tab. 1). The temperature optimum for mycelium growth was near 15°C for the strains from Oberkirch (*Castanea sativa*), Hannover and Poland (*Quercus robur*). In contrast the optimum growth of the other strains was nearly 20°C. None of the strains grew at 30°C and 35°C. To further illustrate the different growth rate of the foreign strains in comparison with one German strain, their mycelium growth is presented in Figure 2 graphically.

The cultures of the 30°C and 35°C variants were transferred to a 20°C climate chamber after eight weeks, but six weeks later it was not possible to detect mycelium growth even when examined with a stereo-microscope. Temperatures above 30°C for the duration of eight weeks were obviously lethal to the fungus. All the other temperature levels with reduced mycelial growth (–3°C, 0°C and 25°C) continued to grow normally when transferred to 20°C.

The results of the average mycelial growth and the statistical comparison of the mean for temperatures frequently occurring during natural or artificial storage of acorns is presented in Figure 3. Significant differences in the average mycelium growth of different provenances were observed at each temperature. The difference between the maximum and the minimum daily average growth was greatest at –3°C and 0°C.

No.	Place of origin	Tree	Year	From
1	Braunschweig[1] D-38165 Wendhausen	*Quercus robur*	1999	Cotyledons
2	Druffelbeck[1] D-38551 Vollbüttel	*Quercus robur*	1999	Cotyledons
3	Poland[3] Miechow	*Quercus robur*	1999	Cotyledons
4	Hanau[1] D-63450 Wolfgang	*Quercus robur*	1999	Cotyledons
5	Müllrose[1, 2] D-15299 Müllrose	*Quercus petraea*	1996	Cotyledons
6	Oberkirch[1], D-77887 Sasbachwalden	*Quercus petraea*	1999	Ascospores
7	Oberkirch (K.)[1], D-77887 Sasbachwalden	*Castanea sativa*	1999	Ascospores
8	Czech Republic[3] 686 04 Kunovice	*Quercus petraea*	1999	Cotyledons
9	Slovenia[1] 1000 Ljubljana	*Quercus robur*	1999	Cotyledons
10	Hannover[1], D-30419 Hannover	*Quercus robur*	1999	Cotyledons
11	Windeck – Schladern[2] D-51570 Windeck	*Quercus rubra*	1989	Cotyledons

Fig. 1. Strains of the fungus *Ciboria batschiana* used in the investigation. (1) fungal strains isolated by the author from freshly collected acorns; (2) fungal strains from the collection of the Institute for Plant Protection in Forests, BBA; (3) pure culture sent from Poland and the Czech Republic

Some strains, for example the one from the Czech Republic, showed a reduced growth rate at nearly all temperatures compared to the others. Others grew near the maximum at every temperature, for example the strain from Müllrose.

To summarize it can be stated that in the present investigation the greatest differences in average mycelium growth were at –3°C, where the fastest-growing strain grew 17 times faster than the slowest-growing strain.

Discussion

The results of the investigation lead to the conclusion that different provenances of *Ciboria batschiana* possess different temperature tolerance particularly at low temperatures near freezing. All strains grew at –3°C and especially the strains from Hanau, Müllrose and Poland showed a high growth rate with an average growth of 0.2 mm at –3°C. Natzke (1999), using the same strain from Müllrose, even realized slight mycelium growth of 0.003 mm per day at a temperature of –10°C. In contrast to these results, Men (1976) reported from France, that the *Ciboria*-strain he tested did not grow below a temperature of 0°C and showed no growth at 27°C. Breisch (1993) reported from France and Italy that *Ciboria batschiana* needs a minimum temperature of 5°C to develop mycelium in stored nuts of *Castanea sativa*. The same temperature level was mentioned by Ridé and Gudin (1969) for a *Ciboria* strain isolated from seeds of *Castanea sativa* in France. Ridé and Gudin established an optimum temperature of 18.5°C and a maximum temperature of 31°C for mycelium growth of *Ciboria batschiana*. In the present investigation both the strains isolated from acorns and from chestnuts showed no mycelium growth at 30°C. However because of the technical equipment it was not possible to test the growth rate in steps between 25°C and 30°C. Hence the exact lethal temperature could not be detected. The differences in lethal temperatures detected by different authors in past studies could well reflect the differences in temperature tolerance between the many European strains of this fungus.

A recent eastward spread of the fungus from France to Poland seems unlikely since strains of *Ciboria batschiana* from France do not show mycelium growth at temperatures lower than 0°C according to Men (1976), Ridé and Gudin (1969) and Breisch (1993). The fungus is definitely able to grow below temperatures of 0°C in the countries tested in this investigation including Poland. Additionally the fungus

Table 1. Average growth of mycelium per day in mm and standard deviation of different *Ciboria batschiana* strains at 9 temperatures; $\bar{x}$ = mean, *s* = standard deviation (n = 10 with 4 measurements each)

Place of Origin		Average daily mycelium growth in mm at x temperature in °C								
		–3	**0**	**5**	**10**	**15**	**20**	**25**	**30**	**35**
Braunschweig	$\bar{x}$	0.15	0.12	1.11	2.31	3.30	3.19	0.26	0.00	0.00
	s	0.06	0.02	0.08	0.17	0.04	0.09	0.07	0.00	0.00
Druffelbeck	$\bar{x}$	0.15	0.57	1.31	2.54	3.28	3.74	0.37	0.00	0.00
	s	0.06	0.02	0.10	0.17	0.30	0.55	0.06	0.00	0.00
Hanau	$\bar{x}$	0.22	0.35	1.21	2.56	3.58	4.03	0.21	0.00	0.00
	s	0.05	0.10	0.08	0.09	0.03	0.09	0.03	0.00	0.00
Hannover	$\bar{x}$	0.02	0.22	0.95	2.06	2.57	1.76	0.27	0.00	0.00
	s	0.01	0.18	0.16	0.18	0.56	0.32	0.07	0.00	0.00
Müllrose	$\bar{x}$	0.20	0.45	1.28	2.78	4.06	4.32	0.44	0.00	0.00
	s	0.04	0.06	0.07	0.15	0.12	0.09	0.09	0.00	0.00
Oberkirch (*Castanea sativa*)	$\bar{x}$	0.13	0.40	1.03	2.13	2.97	2.60	0.27	0.00	0.00
	s	0.04	0.04	0.04	0.14	0.07	0.33	0.03	0.00	0.00
Oberkirch (*Quercus petraea*)	$\bar{x}$	0.06	0.54	1.00	2.09	3.02	3.34	0.32	0.00	0.00
	s	0.02	0.06	0.05	0.13	0.08	0.09	0.03	0.00	0.00
Windeck (*Quercus rubra*)	$\bar{x}$	0.08	0.54	1.06	2.23	3.31	3.30	0.23	0.00	0.00
	s	0.04	0.05	0.05	0.15	0.15	0.12	0.02	0.00	0.00
Poland	$\bar{x}$	0.20	0.43	1.22	2.47	3.13	2.24	0.59	0.00	0.00
	s	0.02	0.05	0.10	0.18	0.24	0.73	0.14	0.00	0.00
Slovenia	$\bar{x}$	0.05	0.19	1.16	2.29	3.00	3.76	0.48	0.00	0.00
	s	0.03	0.05	0.15	0.20	0.29	0.60	0.09	0.00	0.00
Czech Republic	$\bar{x}$	0.01	0.06	0.83	1.72	2.39	2.56	0.21	0.00	0.00
	s	0.01	0.03	0.11	0.26	0.53	0.64	0.02	0.00	0.00

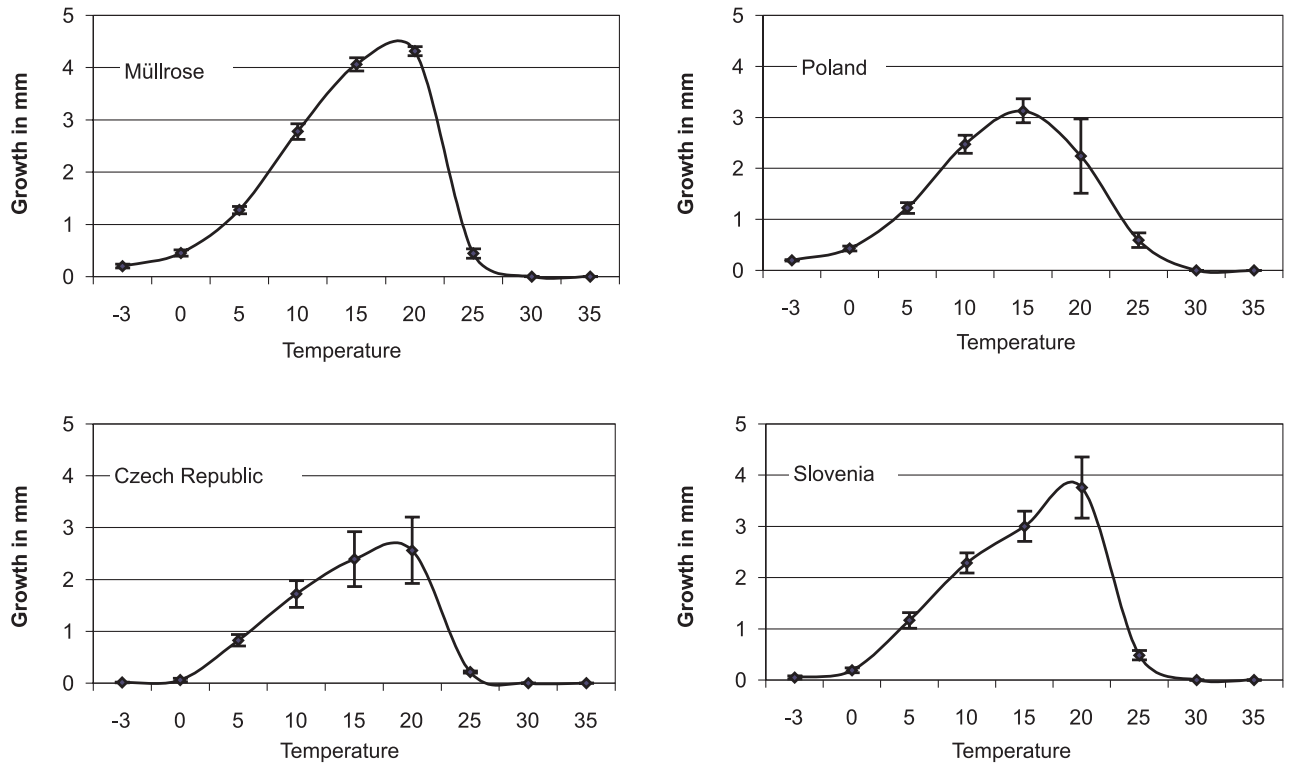

Fig. 2. Average radial growth of mycelium per day of different provenances of the fungus *Ciboria batschiana* at different temperature levels – analyses for provenance. Error bars correspond to the standard deviation at each temperature (n = 10)

has apparently been causing acorn losses during storage for decades even in Russia, Ukraine and the Czech Republic (Sokolov 1955; Urosevic 1959; Zhuravlev and Sokolov 1969; Sokolova and Syemyenkowa 1981; Shevchenko and Tsilyurik 1986; Federov 1987). Therefore it is difficult to reconstruct a possible westward spread of pathogenic strains of the fungus *Ciboria batschiana* in order to explain the relatively recent problems in Poland. In light of the Russian and Ukrainian data, it could be just as likely that the fungus spread westwards from the East to Poland.

The assessment of the recent problems caused by the fungus *C. batschiana* also needs to take into account the possible changes in the environment and changes in acorn processing and storage. The effect of changes in storage methods of acorns on the potential for spread and damage potential of *Ciboria batschiana* should be investigated (for example acorns are no longer stored in underground clamps or mixed with turf, sand or moss). For instance, it seems possible that effective, antagonistic microorganisms which kept *Ciboria batschiana* in check when acorns were stored under natural conditions (in contact with the forest floor or turf or sand), are eliminated by new storage methods without medium in climate chambers.

Several investigations described in recent literature deal with methods for eliminating *Ciboria batschiana* (Delatour 1978; Men 1976; Gille and Nowag 1995; Steinhoff 1993; Schröder 1999). The type size most important examinations were carried out concerning the lethal temperature for the fungus, as influenced by thermal effects. Their effectiveness relies on a temperature-time-relation. Delatour and Morelet (1979) developed a gradient for the lethal temperature on the basis of pure cultures with the following parameters: 48 hours at 36°C, 16 hours at 38°C or 2 hours at 40°C. During the present investigation the influence of 30°C for eight weeks obviously inhibited the fungal growth even in its initial phase and eventually killed the fungus the longer the cultures were exposed to this temperature.

The ability of *Ciboria batschiana* to grow at low temperatures is of great significance for acorn storage because in literature a temperature of –3°C is described as the optimal storage temperature for acorns (Suszka et al. 1996). In many cases this temperature is realized in the storage room but not within the acorns. Using the storage method in open bins developed by Suszka and Tylkowski (1982) the temperature inside the bins, and especially within the acorns, is several degrees above the freezing point for several weeks after the onset of storage in spite of the room temperature of –3°C (Schröder 1999). This is due to the respiratory activity and self-warming of acorns which is especially high during the initial stages of

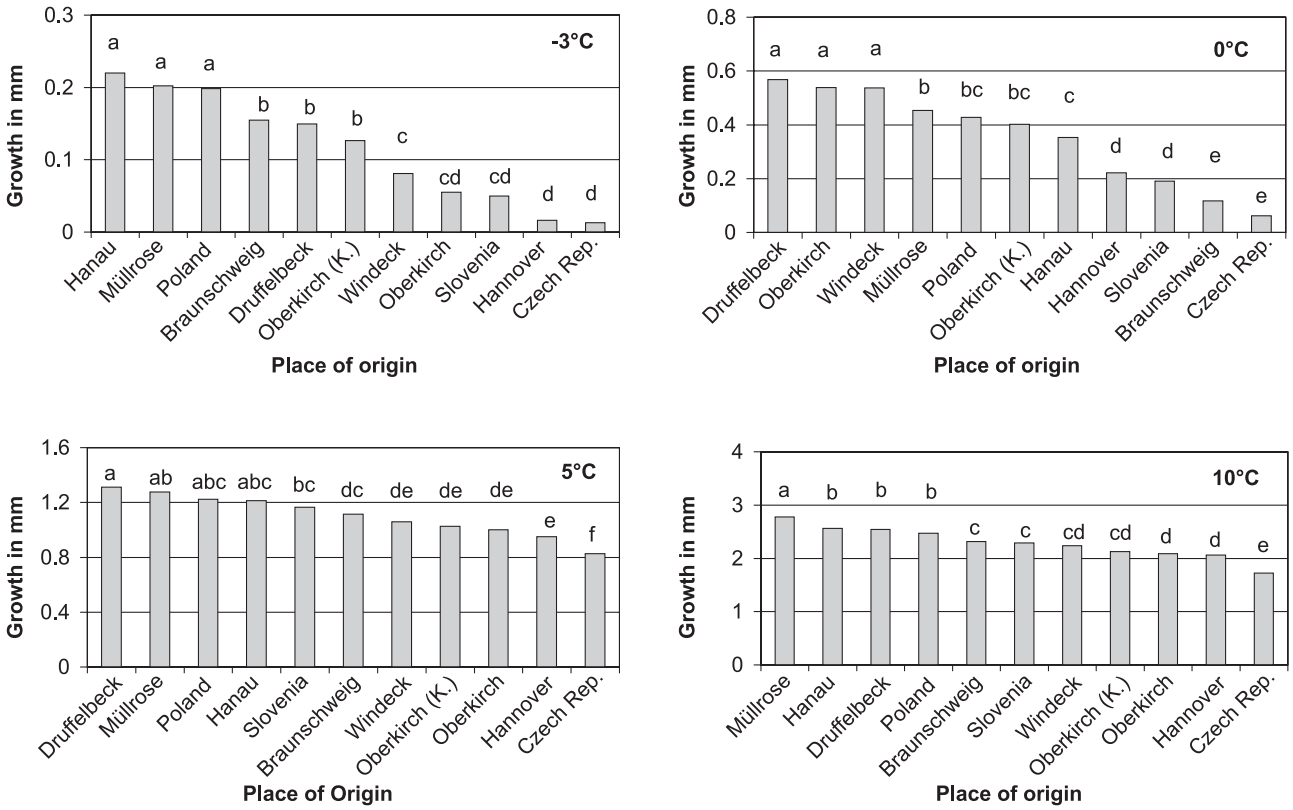

Fig. 3. Average radial mycelium growth per day of eleven fungal strains of *Ciboria batschiana* at four different temperatures – analysis according to temperatures. Different letters show significant differences in mycelium growth of each strain. (Student-Newman-Keuls-Test, $p \leq 0{,}05$; $n = 10$)

storage. In the Forest Seed Centre "La Joux" in France cold air of –7°C is blown into the storage room at the beginning of the storage period to realize a temperature of –1°C in the vicinity of the acorns (Preney 1996, personal communication). In light of the present investigation, these examples show that the fungus finds satisfactory conditions for mycelial growth even under the conditions of "modern" storage techniques. The problem is exacerbated when storing acorns in an atmosphere without frost, thermotherapy and superficial drying prior to storage. Such seed lots are at high risk to be totally destroyed by the fungus *Ciboria batschiana*.

To sum up it can be stated that all tested strains of the fungus *Ciboria batschiana* showed mycelium growth at a temperature of –3°C with an optimum between 15°C and 18°C, making the fungus a representative of the psychrophilous organisms. If an acorn seed lot is infected by *Ciboria batschiana,* storage without prior phytosanitary treatment in the form of "thermotherapy" will lead to the risk of reduced germination capacity or even destruction of the whole seed lot.

Obviously there are significant differences in mycelium growth and frost resistance of the fungus depending on the provenance. This indicates that possibly different "races" or "patho-types" of the fungus exist in Europe, which is a subject for further investigations. In order to enable a precise differentiation between the different geographic origins, more strains from Germany and other European countries should be included in the tests and the main focus should be on molecular biological methods. Testing for differences in pathogenicity of different geographic strains would also be worthwhile but methodically difficult.

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