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MORPHOLOGIAL VARIATION IN COLONIES OF *Chalara fraxinea* ISOLATED FROM ASH (*Fraxinus excelsior* L.) STEMS WITH SYMPTOMS OF DIEBACK AND EFFECTS OF TEMPERATURE ON COLONY GROWTH AND STRUCTURE

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Abstract

The present paper presents the results of long-term observations of differences in growth and morphology of colonies of *C. fraxinea* isolated from ash stems with symptoms of dieback and the results of studies on effects of temperature on growth and morphology of colonies *in vitro*. Thirty randomly chosen *C. fraxinea* cultures, originating from six Forest Districts in southern and northern Poland, viz. Andrychów, Gryfice, Kańczuga, Lębork, Miechów and Mircze, were included in the temperature assay. Colony growth and morphology were evaluated *in vitro,* on malt extract agar. Two replicates of each isolate were incubated at 5, 10, 15, 20, 25 and 30°C in darkness. Colonies of *C. fraxinea* isolated from necrotic stem tissues of diseased ash trees differed greatly in colour, structure, growth rate, presence of sectors and stromata as well as the intensity of conidial sporulation. Colony characters were often lost in further sub-cultures grown on the same medium. Colonies of *C. fraxinea* grew at 5-25°C *in vitro*. Three isolates still grew, though slowly, at 30°C. The optimum temperature for growth was 20°C. Among 30 isolates tested, five grew most quickly at 25°C and four at 15°C. Differences among colony diameters recorded at 5, 10, 15, 20 and 30°C were statistically significant, while differences at 15 and 25°C were not. At some temperatures, statistically significant differences in growth rate were related to the origin of the isolates. There were three main colony colour types. Temperature was the main factor affecting colony morphology *in vitro*. The formation of sectors was observed in 36% of colonies, pseudoparenchymatous stromata in 10.4% of colonies, and white crystalline substances in 53.3% of colonies. The results of *in vitro* observations were used for discussion of the effects of temperature on growth and activity of *C. fraxinea in vivo*.

Key words: *Fraxinus excelsior, Chalara fraxinea*, colonies variability, temperature assay

INTRODUCTION

The studies on the spectrum of fungi involved in dieback of common ash (*Fraxinus excelsior* L.) in Poland resulted in identification and description of the novel fungus *Chalara fraxinea* T. Kowalski (Kowalski, 2006). This fungus was reported from all the forest districts which had been surveyed for the presence of ash trees with dieback symptoms (Kowalski, 2009). The fungus was also found on ash trees with symptoms of dieback in Sweden, Lithuania, Latvia, Denmark, Finland, Germany, Czech Republic, Slovakia, Austria, Hungary, Romania, Slovenia, Switzerland, France, Estonia, and Norway (Zúbrik and Kunca, 2007; Halmschlager and Kirisits, 2008; Jankovsky et al. 2008; Szabo, 2008; Drenkhan and Hanso, 2009; Engesser et al. 2009; Ioos et al. 2009; Kowalski, 2009). Initial studies confirmed that the fungus had significant involvement in the process of ash dieback (Bakys et al. 2009; Kirisits et al. 2009; Kowalski and Holdenrieder, 2009a). Therefore, recognition of conditions which favour its growth is required. So far, little is known on this subject (Kirisits et al. 2008; Kowalski and Holdenrieder, 2009b; Schumacher et al. 2009).

This paper presents the results of long-term observations on differences in morphology and *in vitro* growth of *C. fraxinea* colonies isolated from ash stems with symptoms of dieback, and of studies on effects of temperature on colony morphology and growth.

MATERIALS AND METHODS

Chalara fraxinea was isolated in 2000-2008 from local necrotic lesions and dead stems of ash (*Fraxinus excelsior* L.) trees sampled in different regions of

Poland (Kowalski, 2009). Pieces of stem were surface-sterilized by rinsing in 96% ethanol (1 min), 4% NaOCl (5 min) and 96% ethanol (30 sec). After drying in sterile blotting paper and removing surface bark, 5 x 2 x 2 mm fragments of tissue were cut off and placed in Petri dishes on the surface of 2% malt extract agar (MEA; 20 g l^{-1} malt extract Difco, Sparks, MD, USA, 15 g ´ l^{-1} agar Difco supplemented with 100 mg ´ l^{-1} streptomycin sulphate). Growing mycelium was transferred onto 2% MEA slopes and incubated at 20°C in darkness. The growth and morphological characters of the initial colonies growing from the tissues in different conditions of incubation were observed meanwhile. Numerous sub-cultures of *C. fraxinea* originating from morphologically different colony sectors were made and observed. The outcomes of interactions between colonies of *C. fraxinea* and other fungi isolated at the same time were also recorded. Only the most distinctive morphological characters are described here and used to differentiate colonies of *C. fraxinea*.

Thirty randomly chosen cultures of *C. fraxinea* were included in the temperature assay. They were isolated from ash trees in four Forest Districts in southern Poland, viz. Andrychów (No. 1118, 1122, 1130, 1133, 1135), Kańczuga (1001, 1002, 1004, 1012, 1030), Miechów (625, 627, 628, 631, 1043) and Mircze (721, 725, 726, 732, 738), and two Forest Districts in northern Poland, viz. Lębork (816, 818, 819, 823, 825) and Gryfice (1571, 1573, 1592, 1600, 1603). Inocula used in the temperature assay were produced by growing in Petri dishes on 2% MEA for 4 weeks at 20°C in darkness. Plugs (8 mm diam) taken from the edge of an actively growing colony were transferred onto 2% MEA in Petri dishes. The colonies were incubated for 5 weeks at 5, 10, 15, 20, 25 and 30°C. Growth rate was evaluated on the basis of colony diameter (mm). Two replicates were used for each combination. An average diameter from two measurements in both replicates was estimated. Colony morphology was evaluated on the basis of colour, structure, growth rate, presence of sectors with discoloration, of patchy or linear pseudoparenchymatous stromata and of whitish crystalline agglomerations of secondary metabolites. Three main types of colony colour were distinguished: A – hyaline, evenly white or white with grey patches, or sectors; B - white-orange or white with numerous ferruginous brown patches; C - ferruginous brown. The results for the effects of temperature on growth of *C. fraxinea* were analysed statistically using Duncan's test at significance level $\alpha = 0.05$.

RESULTS

Chalara fraxinea isolated from stems of ash with symptoms of dieback

Chalara fraxinea isolated from necrotic tissues of ash trees stems with symptoms of dieback formed colonies which differed greatly in colour, growth rate and interactions with the colonies of other fungi. Some isolates produced hyaline colonies with thin aerial mycelium, evenly white or white with grey patches or sectors (type A, Fig. 3). The most commonly occurring isolates produced colonies that were cottony, white to orange or white with ferruginous brown patches scattered irregularly on the surface or concentrated around the inoculum (type B, Fig. 1). Other isolates produced ferruginous brown colonies (type C, Fig. 2). Black, oval or irregular pseudoparenchymatous stromata were produced around the inoculum or on the surface of the older, type A colonies (Fig. 3). They were formed less often in the ferruginous coloured colonies (Fig. 4).

One of the most distinctive characters of *C. fraxinea* colonies was a tendency to form sectors that differed in colour, structure of aerial mycelium and growth rate (as shown on Figs 15-17). The characters of the sectors were often lost in sub-cultures grown in new Petri dishes, on MEA, from mycelium transferred from the sectors of the initial colonies.

There was much variation in frequency and density of conidial sporulation among different isolates. Sometimes sporulation occurred only on or around the inoculum (Figs 7, 8). The formation of numerous phialides and phialoconidia was associated with olive grey or dark grey discoloration of the colonies in the sporulation zone. Discoloration was very distinctive in both white (Figs 7, 8) and ferruginous brown colonies (Fig. 9).

Conidial sporulation of *C. fraxinea* was favoured by lower temperatures (5-15°C). The white ferruginous or ferruginous brown, non-sporulating colonies, when incubated at a higher temperature (20°C), started to sporulate abundantly after transfer and further incubation at 5°C. Sporulation appeared along the Petri dish edges in the olive grey discoloured zone (Fig. 10).

The growth rate of *C. fraxinea* colonies was relatively low. During the isolation procedure, *C. fraxinea* colonies were sometimes overgrown by fast growing colonies of other fungi from ash stems. However, the formation of 3–12 mm wide inhibition zones around larger or smaller colonies of *C. fraxinea* was observed more often (Fig. 5). The inhibition zone protected the *C. fraxinea* colony from being overgrown by the fast growing colonies of the species of *Alternaria, Epicoccum, Phomopsis* or *Diplodia*, which were also growing from the ash stems with symptoms of dieback. Agglomerations of white crystalline substances produced during incubation were observed in some *C. fraxinea* colonies (Fig. 6).

Fig. 1.

Fig. 2.

Fig. 1–2. Three-week-old cultures of *C. fraxinea* growing from necrotic tissues of stems of ash with symptoms of dieback (20°C)

Fig. 3.

Fig. 4.

Fig. 3–4. Colonies of *C. fraxinea* with stromata (12 weeks, 15°C)

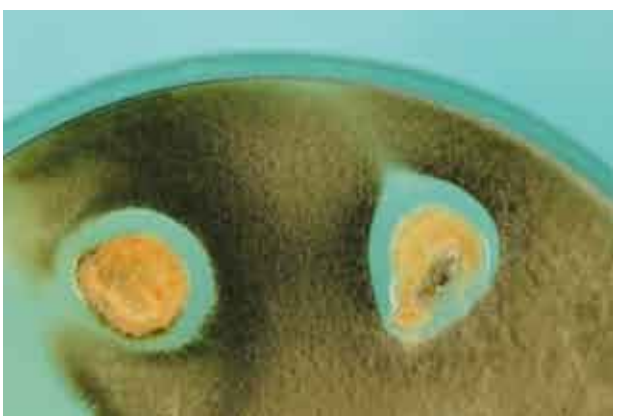

Fig. 5. Inhibition zone between two colonies of *C. fraxinea* and a colony of *Alternaria alternata*

Fig. 6. Colony of *C. fraxinea* with agglomerations of crystalline substances scattered (8 weeks, 20°C)

Fig. 7.

Fig. 8.

Fig. 9.

Fig. 7–9 Five-week-old *C. fraxinea* colonies with local discoloration resulting from sporulation. Fig. 7 – sporulation only on inoculum, 10°C, Fig. 8 – sporulation around inoculum, 5°C , Fig. 9 – sporulation in the form of a ring away from the inoculum, 15°C

Fig. 10. Colony of *C. fraxinea* after incubation for 4 weeks at 20°C followed by 3 weeks at 5°C (olive grey ring results from sporulation)

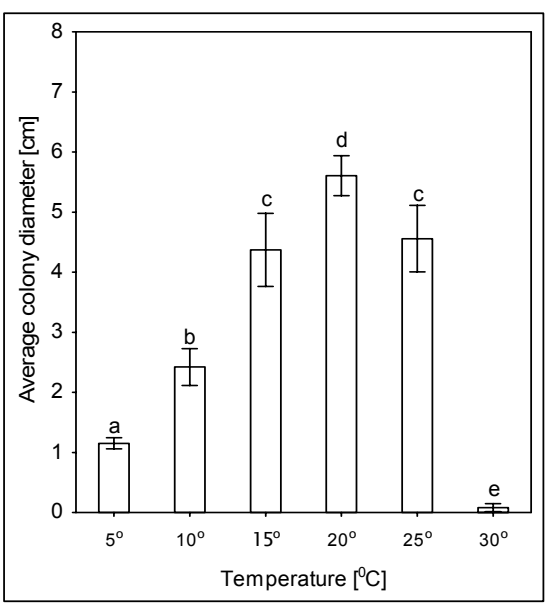

Fig. 11. Growth of *C. fraxinea* colonies *in vitro* in relation to temperature (explanation in table 1)

Temperature assay

The colonies of *C. fraxinea* grew at 5–25°C *in vitro*. Three isolates of the fungus from Gryfice still grew, though slowly, at 30°C (Table 1). Optimum temperature for growth of most isolates was 20°C (Fig. 11). Among 30 isolates tested, five grew fastest at 25°C (Andrychów No. 1122, Mircze No. 726, Gryfice No. 1592, Kańczuga No. 1004, Lębork No. 819) and four at 15°C (Mircze No. 732 and 738, Gryfice No. 1600, Kańczuga No. 1012). Differences among colony diameters recorded at 5, 10, 20 and 30°C were statistically significant; differences recorded at 15 and 25°C were not statistically significant (Fig. 11). At some temperatures, statistically significant differences in growth rate were related to the origin of the isolates (Tab. 1). The smallest differences in growth rate were at 5 and 30°C, where growth was slowest. The fastest average growth occurred at 10°C in isolates from Lębork and Gryfice, at 15°C and 25°C in the isolates from Lębork and Mircze, and at 20°C in isolates from Andrychów, Lębork and Mircze. Significant differences in growth rates among isolates from the same forest district were observed (Tab. 1).

Colony colour of *C. fraxinea* was related to incubation temperature *in vitro*. Type A was predominant at 5 and 10°C (85.0 and 75.0%, respectively), type B at 15 and 20°C (78.3 and 90.0%), and type C at 25°C (66.7%, Tab. 2, Figs 12-14).

Sectors were formed in 36% of colonies, most often at 15°C (11.0%) and most rarely at 5°C (4.0%) (Table 2). In one colony, the sectors differed greatly in colour, structure and growth rate. In the same colony sectors could be ferruginous brown and olive grey (Fig. 15), as well as white with ferruginous brown patches and continuously brown (Fig. 16), or white and dark brown in the ferruginous brown colonies (Fig. 17). Black stromata were formed in 10.4% of colonies, most often at 5 and 10°C (Table 2, Fig. 12). Among 30 isolates tested, 16 produced a whitish crystalline substance located along the entire colony edge, after incubation for 10 weeks at 15 and 20°C, and in two cases at 25°C. Five isolates produced this substance very abundantly.

Fig. 12.

Fig. 13.

Fig. 14.

Fig. 12–14. Different colour types in 5-week-old *C. fraxinea* colonies: Fig. 12 – Type A (10°C), Fig. 13 – Type B (20°C), Fig. 14 – Type C (20°C)

Fig. 15.

Fig. 16.

Fig. 17.

Fig. 15–17. Five-week-old *C. fraxinea* colonies with sectors of different colour, structure and growth rate: Fig. 15 – 5°C, Fig. 16 – 20°C, Fig. 17 –25°C

Table 1
Effect of temperature on *C. fraxinea* growth

Origin	Colony diameter (cm) at temperature											
Forest District	5°C		10°C		15°C		20°C		25°C		30°C	
Andrychów	0,95a*	±0,28**	1,47ab	±0,60	3,46c	±1,17	5,49cd	±1,67	3,61c	±1,42	0,00	
Gryfice	1,68b	±0,56	3,18c	±1,01	4,52cd	±1,88	5,52cd	±0,94	4,90cd	±1,39	0,50e	±0,46
Kańczuga	0,96a	±0,18	1,85ab	±0,55	3,47c	±1,80	3,85c	±0,68	3,04c	±1,23	0,00	
Lębork	1,16a	±0,39	2,89c	±1,20	4,78cd	±1,91	6,82d	±2,04	6,08d	±2,46	0,00	
Miechów	1,00a	±0,19	2,04ab	±0,51	4,18cd	±1,49	5,64cd	±1,77	4,90cd	±1,65	0,00	
Mircze	1,20a	±0,27	3,10c	±1,12	6,16d	±1,67	6,34d	±1,74	4,86cd	±2,09	0,00	

* – Diameters with the same letter do not differ significantly according to Duncan's test ($\alpha = 0.05$)
** – Standard deviation

Table 2
Characters of *C. fraxinea* colonies growing at different temperatures

Temperature	Type of colony (number, %)					Number of colonies
	type resulting from colour*1			colonies with sectors	colonies with stromata	
	A	B	C			
5°C	51 (85,0)	4 (6,7)	5 (8,3)	12 (4,0)	15 (5,0)	60
10°C	45 (75,0)	12 (20,0)	3 (5,0)	19 (6,3)	10 (3,3)	60
15°C	9 (15,0)	47 (78,3)	4 (6,7)	33 (11,0)	2 (0,7)	60
20°C	2 (3,3)	54 (90,0)	4 (6,7)	23 (7,7)	2 (0,7)	60
25°C	0 (0,0)	20 (33,3)	40 (66,7)	21 (7,0)	2 (0,7)	60
Total	107 (35,7)	137 (45,7)	56 (18,6)	108 (36,0)	31 (10,4)	300

*1 – explanations in Materials and Methods

DISCUSSION

The colonies of *C. fraxinea* growing *in vitro* showed considerable variation, particularly in colour, structure, formation of sectors and pseudoparenchymatous stromata. Temperature is one of the main factors that affected colony morphology. The formation of hyaline or white colonies and production of stromata were favoured by lower temperatures, while the formation of more compact, ferruginous brown colonies was favoured by higher temperatures. Colony colour also resulted from the intensity of conidial sporulation, which was favoured by lower temperatures. The colonies became olive grey in the sporulation zones, consistent with observations of Kirisits et al. (2008). Information on morphological variation of *C. fraxinea* colonies can be very useful for quick identification of the pathogen in culture. The formation of sectors in a colony may suggest the presence of more than one fungal species. However, the morphological similarity of sub-cultures growing from inocula taken from particular sectors supports their grouping in a single taxon.

Cardinal temperatures, i.e. minimum, optimum, and maximum, are used to classify fungi as psychrophiles (cold-loving), mesophiles and thermophiles (Griffin, 1981). *Chalara fraxinea* isolated from necrotic ash-stem tissues should be classified as a mesophile, which generally grows best in moderate temperatures (minimum > 0°C, maximum < 50°C, optimum = 15-40°C (Griffin, 1981). Most isolates grew fastest at 20°C. There were, however, isolates which grew fastest at 25°C or 15°C. Different temperature preferences are also shown by isolates of other fungal species (Jung et al. 2001).

Some of the behaviour modes of *C. fraxinea in vitro* may be explained by its biology *in vivo*. Phialides and conidia of *C. fraxinea* are produced *in vivo* on dead ash stems only sporadically (Kirisits et al. 2008; Kowalski, 2009; Kowalski and Holdenrieder, 2009a). They are, however, produced abundantly on the rachises of fallen leaves, in litter, in autumn, at lower temperatures. The black, stromatal layers of mycelium are formed on rachis of fallen leaves. Pseudoparenchymatous stromata formed *in vitro* can be an equivalent of the stromatal layers of mycelium formed *in vivo* (Kowalski, 2006; Kirisits et al. 2009; Kowalski and Holdenrieder, 2009b).

The results of recent studies showed that apothecia of *Hymenoscyphus pseudoalbidus* Queloz et al. (Ascomycota), which was identified as the teleomorph of *C. fraxinea,* are also formed on rachises of fallen leaves (Kowalski and Holdenrieder, 2009b; Queloz et al. 2010). Apothecia appear from July to September. Their occurrence is followed by ascospore discharge, and ascospore germination results in infection of ash trees (Kirisits et al. 2009; Kowalski and Holdenrieder, 2009b).

If the optimum temperature for growth of *C. fraxinea* is usually 20-25°C (only sporadically 15°C), the most favourable conditions for growth *in vivo* occur in summer in the temperate climate of Poland. Colder summer may provide better conditions for isolates with lower temperatures preferences. Extremely hot summers may inhibit the growth of all or most isolates and restrict infection of ash trees. Effects of temperature on growth of the fungus and the effectiveness of infection also occur in other pathogens of forest trees, e.g. *Gremmeniella abietina* (Lagerb.) M. Morelet, *Cenangium ferruginosum* Fr., *Lophodermium seditiosum* Minter, Staley & Millar and *Botrytis cinerea* Pers. (Blenis et al. 1984; Domański and Kowalski, 1988; Butin, 1996).

Some isolates of *C. fraxinea* produced secondary metabolites in the form of white crystalline substances. Grad et al. (2009) found that this substance includes viridin and viridiol. These compounds are closely related: viridiol is a dihydroderivative of viridin (Reino et al. 2008). In nature, the two compounds have different properties. Viridin is highly mycotoxic, with remarkably high activity against certain fungi (Hutchinson, 1999). Viridiol is phytotoxic (Howell and Stipanovic, 1984). Phytotoxic activity of viridiol was observed in ash seedlings, stems and leaves (Anderson et al. 2010; Grad et al. 2009). Viridin seems responsible for creating the inhibition zones around *C. fraxinea* colonies in mixed cultures. The inhibition zone may protect the *C. fraxinea* colony from being overgrown by fast-growing fungi (Fig. 5), Grad et al. 2009; Kowalski and Holdenrieder, 2009a).

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Zróżnicowanie morfologiczne kolonii *Chalara fraxinea* wyizolowanych z jesionów (*Fraxinus excelsior* L.) z objawami zamierania oraz wpływ temperatury na ich wzrost i strukturę

Streszczenie

Przedstawiono wyniki wieloletnich obserwacji nad zróżnicowaniem morfologicznym i rozwojem kolonii *C. fraxinea* bezpośrednio izolowanych z pędów jesionu oraz wyniki badań nad wpływem temperatury na wzrost i zróżnicowanie kolonii *in vitro*. W teście temperaturowym użyto 30 losowo wybranych kultur *C. fraxinea* pochodzących z sześciu nadleśnictw z południowej i północnej Polski: Andrychów, Gryfice, Kańczuga, Lębork, Miechów i Mircze. Wzrost grzybni określano na pożywce agarowo-maltozowej. Dwa powtórzenia każdego izolatu były inkubowane w ciemności w temperaturach: 5, 10, 15, 20, 25 and 30°C. Dodatkowo odnotowywano inne cechy związane z morfologią i rozwojem kolonii *in vitro*.

Kolonie *C. fraxinea* wyrastające z nekrotycznych tkanek na pędach jesionu cechowała duża zmienność w zakresie barwy, tempa wzrostu, wykształcania sektorów, tworzenia stromata oraz zarodnikowania. Cechy kolonii stosunkowo często nie były odtwarzane w subkulturach hodowanych na tej samej pożywce.

Kolonie *C. fraxinea* wykazywały *in vitro* wzrost w zakresie temperatur od 5 do 25°C, a trzy izolaty wzrastały minimalnie także w 30°C. Optimum wzrostu dla większości szczepów wynosiło 20°C. Spośród testowanych 30 izolatów, pięć rosło najszybciej w 25°C, a cztery w 15°C. Stwierdzono statystycznie istotne różnice pomiędzy średnicami kolonii w temperaturze 5, 10, 15, 20 i 30°C oraz brak takich różnic w 15 i 25°C. W niektórych temperaturach stwierdzono statystycznie istotne różnice w tempie wzrostu kolonii zależnie od miejsca ich pochodzenia. Najmniejsze zróżnicowanie było w tym zakresie w temperaturach 5 i 30°C, w których wzrost szczepów był najwolniejszy.

Wyróżniono trzy zasadniczo różniące się typy barwne kolonii, których częstość występowania zależała w znacznym stopniu od temperatury hodowli *in vitro*. Tworzenie sektorów zostało zaobserwowane u 36% kolonii, wykształcanie pseudoparenchymatycznych stromata u 10.4%, a obecność krystalicznej substancji u 53.3% kolonii. W dyskusji podjęto próbę odniesienia się do warunków rozwoju sprawcy zamierania jesionu *in vivo* w świetle uzyskanych wyników *in vitro*.