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THE EFFECT OF WATER ACTIVITY AND TEMPERATURE ON THE GROWTH AND LIPOLYTIC ACTIVITY OF *PENICILLIUM CHRYSOGENUM* (THOM) AND *EUROTIUM HERBARIORUM* (WIGG.) STRAINS

WPŁYW AKTYWNOŚCI WODY I TEMPERATURY NA WZROST ORAZ AKTYWNOŚĆ LIPOLITYCZNĄ SZCZEPÓW *PENICILLIUM CHRYSOGENUM* (THOM) I *EUROTIUM HERBARIORUM* (WIGG.)

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Streszczenie. Określono wpływ aktywności wody (a_w) i temperatury powietrza na wzrost i hydrolizę tributyryny szczepów *P. chrysogenum* (Thom) i *E. herbarioroum* (Wigg.), które wyizolowano z nasion rzepaku, soi i słonecznika. Na podstawie otrzymanych wyników stwierdzono, że badane szczepy charakteryzowały się zróżnicowaną, w zależności od a_w pożywki i temperatury inkubacji, zdolnością wzrostu na podłożu z tributyryną. Wykazano także różnice w dobowych przyrostach promienia kolonii, stref hydrolizy tributyryny oraz we wskaźnikach hydrolizy tributyryny pomiędzy szczepami tego samego gatunku, w zależności od tego, z jakich nasion zostały wyizolowane. Szczepy *E. herbarioroum*, w odróżnieniu od szczepów *P. chrysogenum*, nie rosły na podłożu z tributyryną w 15°C, przy a_w 0,850. W tych warunkach nie wykazano także strefy hydrolizy substratu tłuszczowego. Większe dobowe przyrosty promienia strefy hydrolizy tributyryny i promienia kolonii na pożywce TBA stwierdzono w przypadku szczepów obu rodzajów w temperaturze 25°C. Wyraźne różnice pomiędzy *E. herbariorum* a *P. chrysogenum* zaobserwowano we wskaźnikach hydrolizy tributyryny.

Słowa kluczowe: tributyryna, aktywność wody, aktywność lipolityczna, *Penicillium chrysogenum, Eurotium herbariorum.*

Key words: tributyrin, water activity, lipolytic activity, *Penicillium chrysogenum, Eurotium herbariorum.*

INTRODUCTION

Owing to their ability to grow and develop in various, often extreme, environmental conditions, filamentous fungi pose problems in many aspect of human life and industrial activity being responsible for deterioration in the quality standards of raw materials and food products resulting in substantial economic losses (Cole et al. 1976; Adamczak and Bednarski 1996; Kacaniova 2003; Baydar and Erbaş 2005; De Lucca 2007; Dagnas and Membré 2013; Prusak et al. 2014).

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Adverse effects of microorganisms are also observed in the seeds of oil crops (El-Kady and Youssef 1993; Bielecka et al. 1995; Filtenborg et al. 1996; Bhattacharya and Raha 2002; Nasir 2003; Sariyar and Heperkan 2003; Gruzdeviene et al. 2006; Sharfun-Nahar Mushtaq and Hashmi 2005). Problems are posed by seed borne mycoflora during long-term storage. Microorganisms used the most available chemical compound which can be used as carbon source, which in oil crops are vegetable oils. When exposed to inappropriate storage conditions, oil crop seeds can be attacked by a variety of microorganisms. Xerophilic and xerotolerant fungi with lipolytic properties play a major role in the biodeterioration of oil crops (Hadanich et al. 2008).

The literature contains only one paper on the effect of water activity and temperature on the growth and lipolytic activity of fungi separated from moldy rape seeds, including *P. chrysogenum* (Magan et al. 1993). There are no available reports on the lipolytic activity of *E. herbariorum* and *P. chrysogenum* strains.

Due to high extracellular complex of an enzyme production, mould fungi have been implicated in the quality deterioration of many vegetable oils and oil seed. In order to prevent the development and spread of spoilage to define the factors which influenced on that process is needed. The aim of the study was to determine the effect of water activity and temperature on the growth and lipolytic activity of *P. chrysogenum* (Thom) and *E. herbariorum* (Wigg.) strains isolated from the seeds of oil crops.

MATERIAL AND METHODS

Esterase hydrolytic activity was studied on TBA − tributyrin agar (Magan et al. 1993). Depending on sodium chloride concentration, media with various water activity of 0.995; 0.950; 0.900; and 0.850 were obtained (Lang 1967). Water activity of the media was verified using a measuring unit manufactured by Decagon: DE 202 Aqua Lite. Five strains of *P. chrysogenum* (Thom) and 5 strains of *E. herbariorum* (Wigg.) isolated from rape and soya-bean as well as 4 strains of *P. chrysogenum* and 4 strains of *E. herbariorum* isolated from sunflower (a total of 28 strains) were used. Spore suspensions were prepared by adding physiological salt solution (NaCl). To obtain suspensions, strain cultures were obtained on MEA slopes (Merck Microbiology Manual, 2006) at 25°C. For fast growing *Penicillium* strains, 5−7 day fungal cultures were used, whereas for slow growing *Eurotium* strains, 14-day cultures were applied. Slopes were filled with sterile dilution liquid (7 cm^3) and shaken for 1 min. Depending on the strain and fungal species, the number of spores in suspension varied in a range from 10^6 to 10^7 in 1 cm^3. Fungal culture tests, which were repeated three times, were performed by placing 5 µl of spore suspension in the middle of each Petri plate with TBA medium. The cultures were kept at 15°C and 25°C for 30 days. The radius values of colonies and tributyrin hydrolysis zones (visible light shades of nutrient around fungal colonies) were measured every 5 days. Daily growth rates of the colony radius and tributyrin hydrolysis radius were determined using linear regression:

$$r = a \cdot t + b$$

where:

r — radius of colony or tributyrin hydrolysis zone [$mm \cdot day^{-1}$],

b — growth rate retardation, lag phase λ coefficient.

According to Dantigny et al. (2005), coefficient b does not make any biological sense, since all calculations are based on macroscopic observations of fungal growth. Therefore, coefficient b was not considered in the present study. The coefficient of tributyrin hydrolysis was calculated, expressed as the ratio of daily growth rates of hydrolysis zone to daily growth rates of colony radius. Statistical analysis of obtained results was conducted using Excel spreadsheet and Statistica 8.0. For statistical significance analysis, analysis of variance (ANOVA) was used for $p \leq 0.05$.

RESULTS

The results of daily growth rates for *P. chrysogenum* strains are presented in Fig. 1. All the strains grew the fastest at 0.995 a_w. The values were: for rape strains 1.1 and 1.31, for soya-bean strains 1.21 and 1.63 and for sunflower strains 1.1 and 1.36 mm · day^{-1}, at 15°C and 25°C, respectively. The higher was a_w, the larger the daily growth rates tended to be.

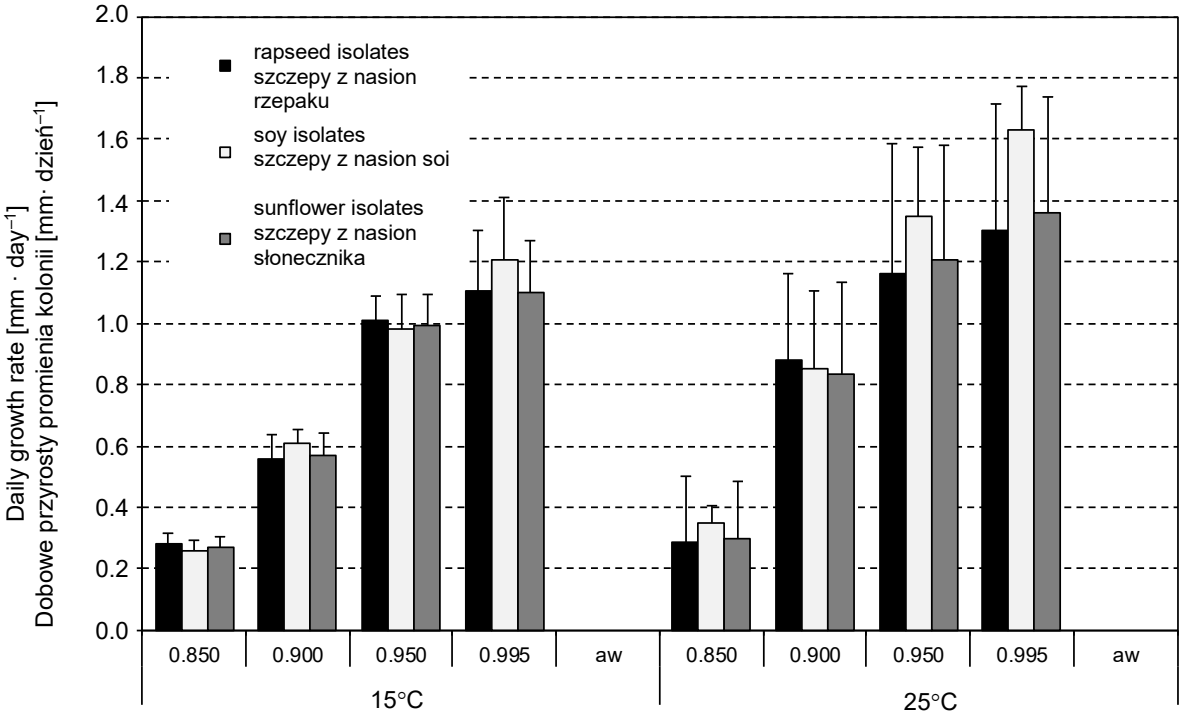

Fig. 1. Average daily growth rates of colony radius of rape, soya-bean and sunflower *P. chrysogenum* (Thom) strains
Ryc. 1. Średnie dobowe przyrosty promienia kolonii szczepów *P. chrysogenum* (Thom) wyodrębnionych z nasion rzepaku, soi i słonecznika

The daily growth rates of tributyrin hydrolysis zones for *P. chrysogenum* strains are presented in Fig. 2. The rape and sunflower strains reached their highest values at 15°C and 0.950 a_w (1.07 and 1.17, respectively) and at 25°C and 0.995 a_w (1.59 and 1.6 mm · day^{-1},

respectively). The soya-bean strains had the highest daily growth rates at 0.950 a_w (1.38 mm · day^{-1} at 15°C and 1.79 mm · day^{-1} at 25°C). For 0.850 and 0.900, the daily growth rates of hydrolysis zone radius of all the strains for both temperatures were similar. For 0.950 and 0.995 a_w, the daily growth rates of tributyrin hydrolysis radius were higher for strains kept at 25°C.

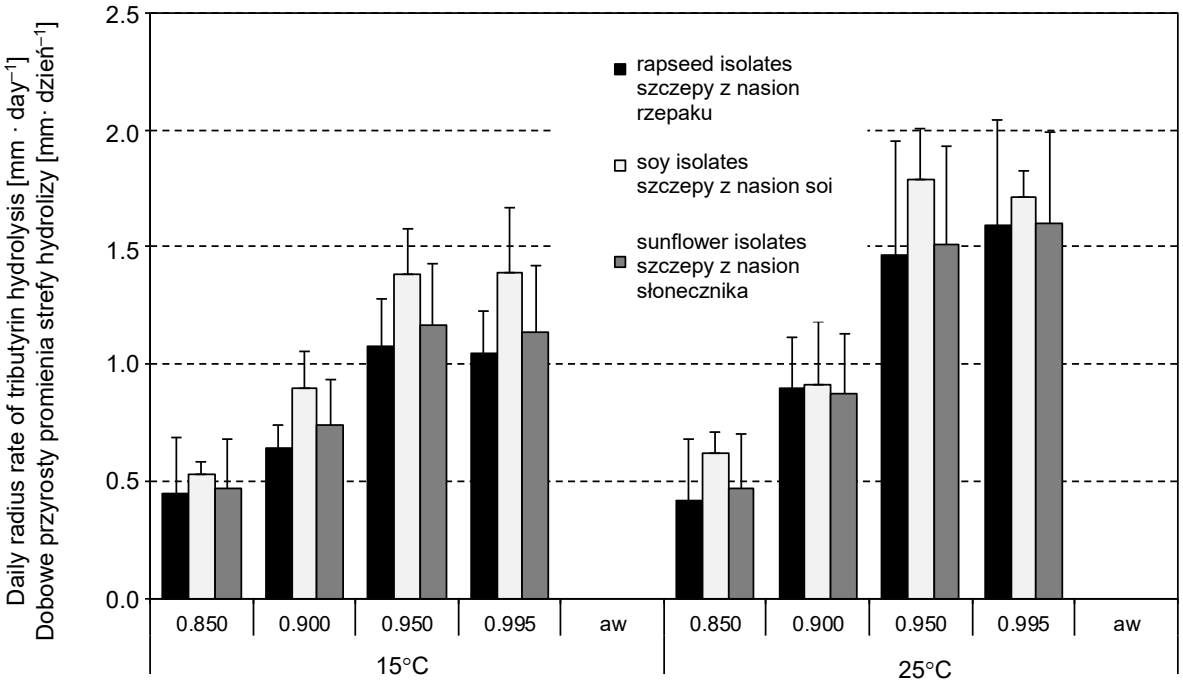

Fig. 2. Average daily growth rates of tributyrin hydrolysis zone of rape, soya-bean and sunflower *P. chrysogenum* strains
Ryc. 2. Średnie dobowe przyrosty promienia strefy hydrolizy tributyryny podczas hodowli szczepów *P. chrysogenum* wyodrębnionych z nasion rzepaku, soi i słonecznika

The coefficients of tributyrin hydrolysis for *P. chrysogenum* strains are presented in Fig. 3. All the strains had their highest tributyrin hydrolysis coefficients at 0.850 a_w. The values were: for rape strains 1.59 and 1.47, for soya-bean strains 2.02 and 1.77 and for sunflower strains 1.72 and 1.55 mm · day^{-1}, at 15°C and 25°C, respectively. Regardless of the strain origin, somewhat higher tributyrin hydrolysis coefficients were observed at 15°C.

The rape strains had their highest daily growth rates at 0.950 a_w (0.67 at 15°C and 1.08 mm · day^{-1} at 25°C). The soya-bean strains had their highest rates at 15°C and 0.995 a_w, and at 25°C and 0.950 a_w (1.1 and 1.34 mm · day^{-1}, respectively). The sunflower strains, like the rape strains, had their highest growth rates at 0.950 a_w (0.87 and 1.55 mm · day^{-1}, at 15°C and 25°C, respectively).

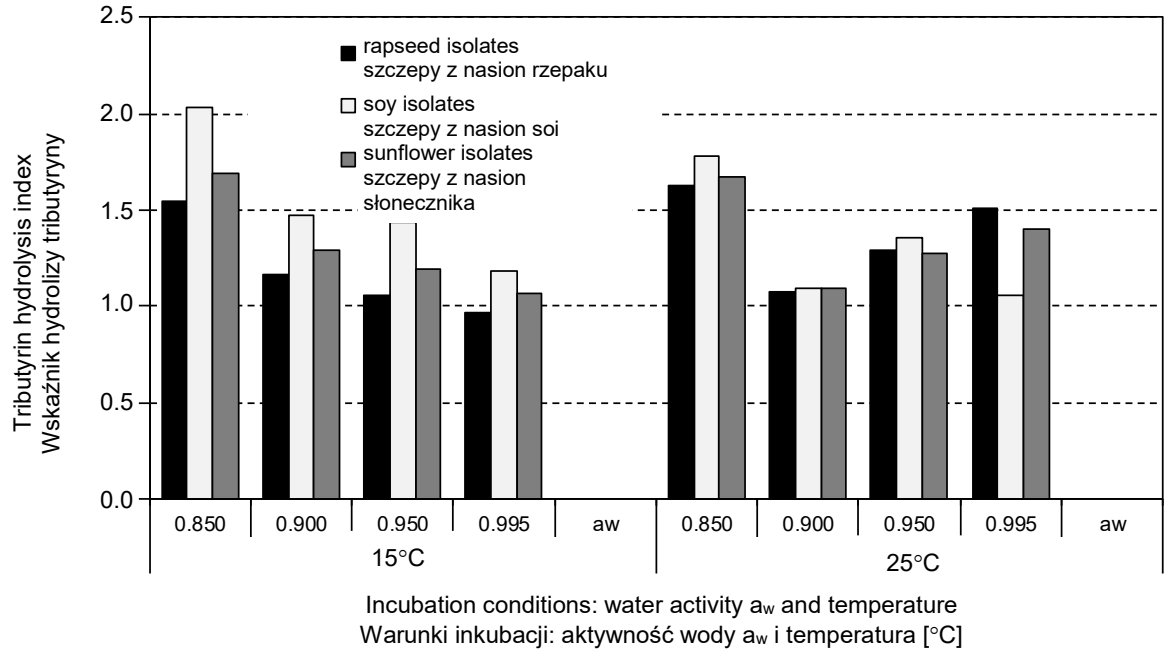

Fig. 3. Tributyrin hydrolysis coefficients of rape, soya-bean and sunflower *P. chrysogenum* strains
Ryc. 3. Wskaźniki hydrolizy tributyryny uzyskane dla szczepów *P. chrysogenum* wyodrębnionych z nasion rzepaku, soi i słonecznika

The results of daily growth rates for *E. herbariorum* strains are presented in Fig. 4. No growth was observed, in any strains, at 15°C and 0.850 a_w. No growth of the soya-bean strains was observed at 25°C and 0.850 a_w. Little growth of the rape and sunflower strains was observed at 25°C and 0.850 a_w (0.06 and 0.05 mm · day^{-1}, respectively).

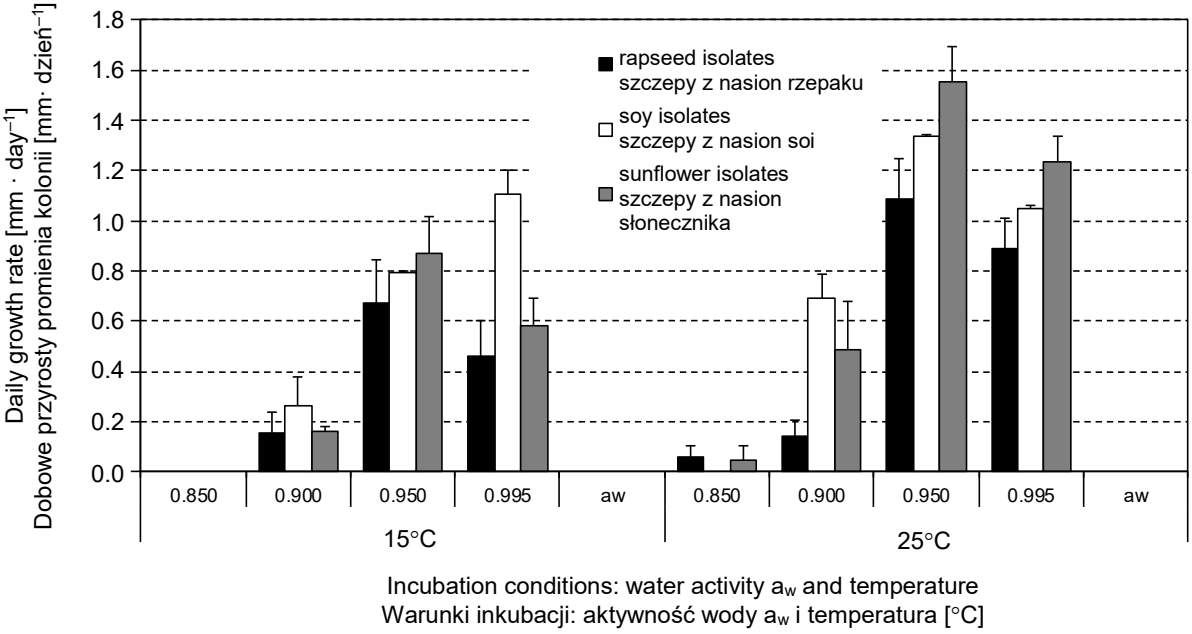

Fig. 4. Average daily growth rates of colony radius of rape, soya-bean and sunflower *Eurotium herbariorum* (FH Wigg.) strains
Ryc. 4. Średnie dobowe przyrosty promienia kolonii szczepów *Eurotium herbariorum* (FH Wigg.) wyodrębnionych z nasion rzepaku, soi i słonecznika

The daily growth rates of tributyrin hydrolysis zones for *E. herbariorum* strains are presented in Fig. 5

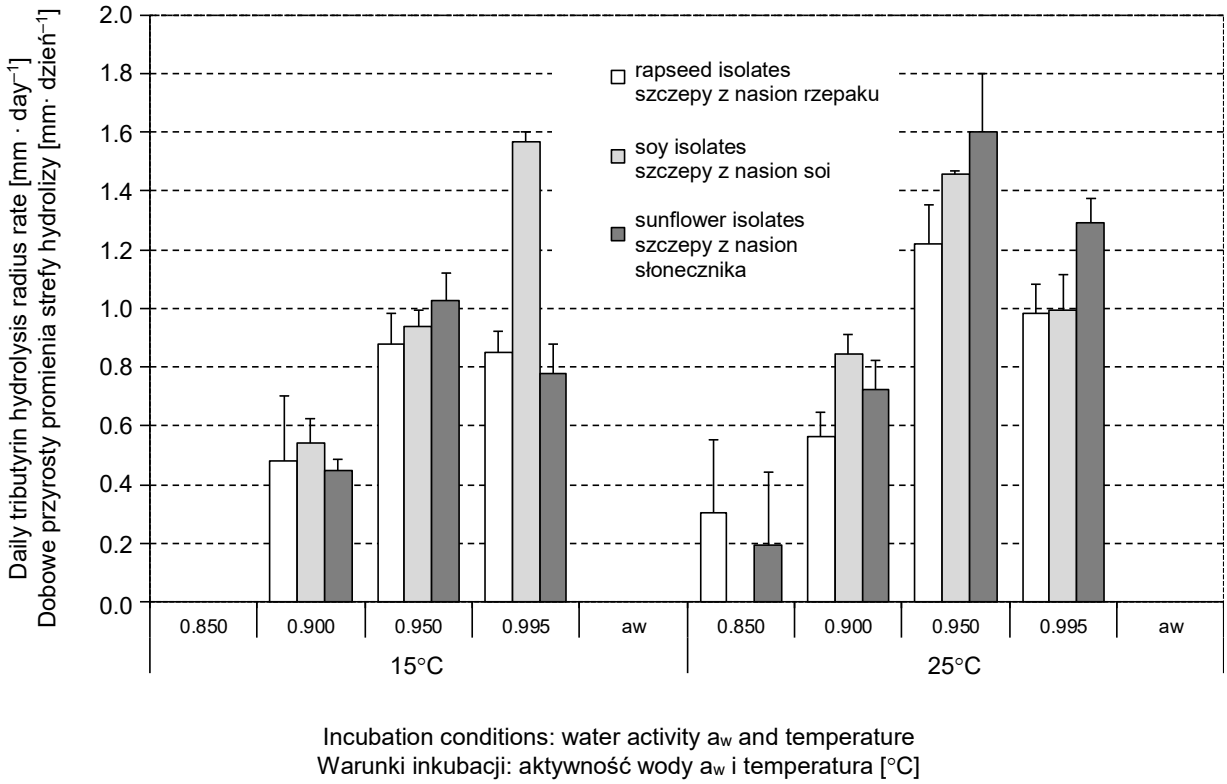

Fig. 5. Average daily growth rates of tributyrin hydrolysis zone of rape, soya-bean and sunflower *E. herbariorum* strains
Ryc. 5. Średnie dobowe przyrosty promienia strefy hydrolizy tributyryny podczas hodowli szczepów *E. herbariorum* wyodrębnionych z nasion rzepaku, soi i słonecznika

The rape strains had the highest daily growth rates of hydrolysis zone radius at 15°C and 25°C and 0.950 a_w (0.88 and 1.22, respectively). The soya-bean strains had the highest daily growth rates at 15°C at 0.995 a_w (1.57 mm · day^{-1}), and at 25°C at 0.950 a_w (1.46 mm · day^{-1}). The sunflower strains, like rape strains, had the highest daily growth rates of hydrolysis zone radius at 15°C and 25°C and 0.950 a_w (1.02 and 1.6 mm · day^{-1}, at 15°C and 25°C, respectively). No hydrolysis zones were distinguished at 15°C and 0.850 a_w, regardless of *E. herbariorum* strain type. No hydrolysis zones were observed at 25°C in the soya-bean strains only. At 15°C, the highest daily growth rates of hydrolysis zones were observed at 0.995 a_w in the soya-bean strains (1.57 mm · day^{-1}). On the other hand, at 25°C, the highest rates were observed at 0.950 a_w in sunflower strains (1.60 mm · day^{-1}).

The coefficients of tributyrin hydrolysis for *E. herbariorum* strains are presented in Fig. 6.

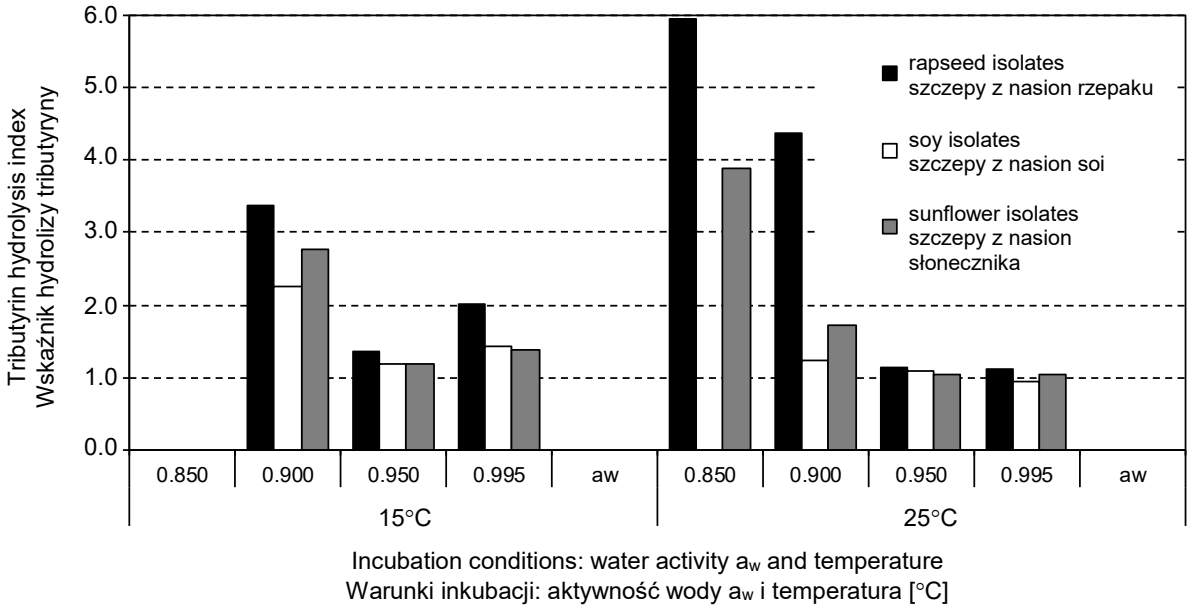

Fig. 6. Tributyrin hydrolysis coefficients of rape, soya-bean and sunflower *E. herbariorum* strains
Ryc. 6. Wskaźniki hydrolizy tributyryny uzyskane dla szczepów *E. herbariorum* wyodrębnionych z nasion rzepaku, soi i słonecznika

The rape strains demonstrated the highest hydrolytic activity at 15°C and 0.900 a_w (3.11) and at 25°C and 0.850 a_w (5.32). The soya-bean strains had the highest coefficients of tributyrin hydrolysis at 0.900 a_w (2.74 and 1.23, at 15°C and 25°C, respectively). The sunflower strains, like the rape strains, showed the highest activity at 15°C and 0.900 a_w and at 25°C and 0.850 a_w (2.74 and 4.09, respectively). No lipolytic activity was observed at 25°C for the soya-bean strains only. The highest coefficient of tributyrin hydrolysis (5.32) was observed for the rape strains at 25°C and 0.850 a_w. The lowest levels of lipolytic activity were detected at 25°C and 0.950 and 0.995 a_w; depending on strain origin the values varied from 1.13 to 0.95.

Both *P. chrysogenum* (Table 1) and *E. herbariorum* (Table 2) strains, the daily growth rates of colony radius, the daily growth rates of tributyrin hydrolysis zone and the tributyrin hydrolysis coefficients were dependent mostly on water activity a_w.

Table 1. Analysis of variance (ANOVA) for growth coefficients and tributyrin hydrolysis by *Penicillium chrysogenum* (Thom) strains
Tabela 1. Analiza wariancji (ANOVA) dla współczynników wzrostu i hydrolizy tributyryny przez szczepy *Penicillium chrysogenum* (Thom)

Factors Czynniki	Daily growth rate of tributyrin hydrolysis radius Dobowy przyrost promienia strefy hydrolizy tributyryny		Daily growth rate of colony radius Dobowy przyrost promienia kolonii		Tributyrin hydrolysis coefficient Wskaźnik hydrolizy tributyryny	
	F	p	F	p	F	p
Temperature Temperatura	15.98	0.0001*	14.55	0.0002*	0.00	0.9587
Water activity Aktywność wody	111.83	0.0000*	161.12	0.0000*	12.54	0.0000*
Strain isolation origin Źródło izolowania szczepów	7.69	0.0061*	1.19	0.2764	3.99	0.0470*

* For statistical significance level of $p \leq 0.05$ – zależności istotne na poziomie istotności $p \leq 0,05$.

Table 2. Analysis of variance (ANOVA) for growth coefficients and tributyrin hydrolysis by *Eurotium herbariorum* strains
Tabela 2. Analiza wariancji (ANOVA) dla współczynników wzrostu i hydrolizy tributyryny przez szczepy *Eurotium herbariorum* (FH Wigg.)

Factors Czynniki	Daily growth rate of tributyrin hydrolysis radius Dobowy przyrost promienia strefy hydrolizy tributyryny		Daily growth rate of colony radius Dobowy przyrost promienia kolonii		Tributyrin hydrolysis coefficient Wskaźnik hydrolizy tributyryny	
	F	p	F	p	F	p
Temperature Temperatura	17.65	0.0000*	23.25	0.0000*	0.42	0.5186
Water activity Aktywność wody	200.12	0.0000*	152.87	0.0000*	43.84	0.0000*
Strain isolation origin Źródło izolowania szczepów	2.77	0.0652	7.18	0.0010*	8.50	0.0003*

*For statistical significance level of $p \leq 0.05$ – zależności istotne na poziomie istotności $p \leq 0,05$.

DISCUSSION

The effect of water activity combined with environmental factors such as temperature, pH and sodium chloride concentration, on the growth and enzymatic activity of microorganisms has been the focus of many studies. Cuppers et al. (1997) proposed a model of how temperature and NaCl affect the growth of *Penicillium roqueforti* (Thom), *Trichoderma harzianum* (Rifai), *Paecilomyces variotii* (Brown & Smith), *Aspergillus niger* (Tiegh) and *Emericella nidulans* (Winter), responsible for food spoilage. Gock et al. (2003) studied the effect of water activity, temperature and pH on the growth of xerophilic fungi *Eurotium rubrum* (Konig), *E. repens* (Blochwitz), *Wallemia sebi* (Fries), *Aspergillus penicillioides* (Speg.), *Penicillium roqueforti* (Thom), *Chrysosporium xerophilum* (Pitt) and *Xeromyces bisporus* (Fraser). Other mould species, i.e. (*Aspergillus candidus,* Link*, Aspergillus niger,* van Tieghem*)* and *Penicillium implicatum* (Biourge) were examined by Cahagnier et al. (2008) and Parra and Magan (2004).

Sautour et al. (2002) and Rosso and Robinson (2001) presented models which described the effect of water activity on fungal growth. However, the authors did not study the role of the above-mentioned factor in fungal lipolytic activity. This activity was the focus of the study by Magan et al. (1993), who in this aspect investigated *Aspergillus*, *Eurotium* and *Penicillium* strains isolated from rapeseeds. The researchers demonstrated that there was no direct relationship between colony growth and strain lipolytic activity on TBA. They proved that optimum conditions of fungal growth did not always overlap with optimum conditions of lipase biosynthesis. They also found that while *Penicillium* strains were more active and increased hydrolytic activity was observed on TBA at 15°C, *Aspergillus* strains were more active at 25°C. The authors speculated that the lack of colony growth or lipolytic activity of *Penicillium chrysogenum* at 0.850 a_w could be caused by very retarded germination of spores under such conditions. The phenomenon might also be due to the fact that such a level of a_w is close to that of the minimum value, necessary to trigger germination of most species of *Penicillium*. The hypothesis was confirmed in a study by Plaza et al. (2003). According to these authors, temperature and a_w are major abiotic factors determining spore germination. Their findings show that the more temperature and a_w values differ from optimum values, the

longer the lag phase and the slower colony growth tend to be. Pardo et al. (2006) also indicated temperature and a_w as the decisive abiotic factors affecting fungal growth and germination. They proved that lower values of a_w led to decreased germination of *Penicillium verrucosum* (Dierckx.) spores. Magan and Lacey (1988) demonstrated that a lengthening of the lag phase of *Aspergillus echinulatus* (Delacr.) spore germination was associated with decreased levels of a_w. Magan et al. (1993) claimed that whereas *Penicillium* strains play a pivotal role as fungi triggering rape spoilage in low temperatures, *Aspergillus* strains become more prominent in this role at 25°C. Their findings demonstrated that *Eurotium amstelodami* (L. Mangin) strains had little ability to hydrolyze rape oil on agar medium, regardless of a_w. It seems that the species plays a fundamental role in rape storage, particularly in a temperature range from 20°C to 30°C. However, fungi growing in the mentioned temperature even at low a_w values, do not display lipolytic activity. The authors also pointed out that fungal growth and their biodeterioration activity were both dependent on interactions between different fungal species. They argued that the rape seed borne fungi produce the enzymes responsible for degradation of rape seeds or pressed oil. Our study suggests that this is also the case with both sunflower and soya-bean seed borne mycoflora.

Our studies confirmed earlier findings of Magan et al. (1993) who reported that optimum conditions of fungal growth did not always overlap with optimum conditions of lipases biosynthesis. The present study demonstrated that *P. chrysogenum* strains, contrary to Magan's et al. (1993) findings, had the ability to grow and hydrolyze tributyrin at 0.850 a_w. Our findings were consistent with those obtained by Sathya et al. (2009). They claim that although air humidity and temperature are major abiotic factors responsible for seed deterioration, humidity plays the paramount role and elevated temperature plays a secondary role in the above-mentions process. Skiba et al. (2005) maintains that an increase of temperature from 15°C to 25°C causes a four-fold raise in the intensity of biochemical processes. Interestingly, a humidity increase from 7% to 9% causes a staggering eight-fold increase in the intensity of these processes.

Our experiments showed that *P. chrysogenum* strains in both temperatures had larger colony growth at 0.950 and 0.995 a_w and the largest coefficient of tributyrin hydrolysis at 0.850 a_w. Likewise, *E. herbariorum* strains, both at 15°C and 25°C, grew better at 0.950 and 0.995 a_w, although had higher tributyrin hydrolysis coefficients at 0.900 and 0.850 a_w (at 15°C and 25°C, respectively). The problem needs to be further investigated, though, since findings obtained in fungal cultures on TBA cannot be directly extrapolated to seed storage conditions.

CONCLUSIONS

In this study, mould fungi being responsible for biodeterioration of soya-bean, sunflower and rapeseed seeds, i.e. *Penicillium chrysogenum* (Thom) and *Eurotium herbariorum* (Wigg.) were examined. Three main observations of the study are as follows:

1. *E. herbariorum* neither grows nor hydrolyzes tributyrin on a solid medium at 15°C and 0.850 a_w. At this temperature, the highest coefficient of tributyrin hydrolysis was recorded at 0.900 a_w. The lipolytic activity of *E. herbariorum* at 0.850 a_w was stimulated at 25°C.

2. *P. chrysogenum* reached its highest tributyrin hydrolysis coefficients at the lowest values of a_w (0.850) at 15°C.
3. The daily growth rates of hydrolysis zone radius and tributyrin hydrolysis coefficient values of *P. chrysogenum* and *E. herbariorum* were found to be mostly dependent on a_w of the medium.
4. All detected relationships between the fungal growth an water activity and temperture might be used for improving grain storage and prevention of fungal proliferation. A presented data has considerable potential for reducing seed storage losses

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Abstract. The aim of the study was to determine the effect of water activity (a_w) and temperature on the growth and tributyrin hydrolysis of *Penicillium chrysogenum* and *Eurotium herbariorum* strains isolated from rape, soya-bean and sunflower seeds. The study demonstrated differences in daily growth rates, tributyrin hydrolysis zones and tributyrin hydrolysis indices in different strains of the same species; depending on the seeds they had been isolated from. *E. herbariorum* strains, unlike *P. chrysogenum* strains, did not grow on tributyrin at 15°C and 0.850 a_w. No hydrolysis zones of oil substrate were found under these conditions. *E. herbariorum* and *P. chrysogenum* showed marked differences in their tributyrin hydrolysis indices. For *P. chrysogenum* strains somewhat higher values were recorded for cultures at 15°C. However, the highest indices of tributyrin hydrolysis at 15°C and 25°C were recorded at the lowest level of a_w 0.850.