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Keywords: cellulases, fungal cultivation, growth rate of mycelium, ligninases, phytopathogens, pure cultures, xylotrophic fungi.

Сравнительное изучение ростовых параметров и лигноцеллюлолитической активности
dевяти штаммов Sarcodontia crocea на четырёх разных питательных средах

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A COMPARATIVE STUDY ON GROWTH AND LIGNOCELLULOLYTIC ACTIVITY
OF NINE SARCORDONTIA CROCEA STRAINS IN FOUR DIFFERENT MEDIA

© S. V. Volobuev, N. V. Shakova

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Аннотация. Представлены результаты изучения ростовых характеристик, макроморфологических признаков
и биосинтетического потенциала девяти дикариотических штаммов Sarcodontia crocea (Polyporales, Basidiomycota), хра-
нящихся в Коллекции культур базидиомицетов БИН РАН (LE-BIN). Исследованные штаммы были получены из базиди-
ом, собранных на Malus domestica в Белгородской, Орловской и Ростовской областях, а также путем высева базидиоспор.
Культурально-морфологические признаки и ферментативная активность S. crocea исследованы при выращивании штаммов
как на стандартных питательных средах (малы-экстракт агаре – МЭА, глюкозо-пептонном агаре – ГПА), так и на
модифицированных полусинтетических агаризованных средах. Разработан оригинальный состав и впервые апробированы
питательные среды, приготовленные с использованием водных экстрактов древесины Malus domestica (Malus-M) и
Pyrus communis (Pyrus-M). Установлено, что при культивировании S. crocea на агаризованных питательных средах
ГПА, Malus-M и Pyrus-M наблюдалось существенное снижение скорости роста. При выращивании исследуемых штаммов
на Malus-M и Pyrus-M наблюдалась значительная вариабельность колоний, разреженность мицелиального мата,
а также потеря зональности и выраженной воздушной мицелия, по сравнению с МЭА. Показано, что состав питатель-
ной среды существенно определял способность штаммов S. crocea к продукции ферментов лигноцеллюлолитического
комплекса. Целлюлолитическая активность была отмечена для штаммов на всех исследуемых средах, при этом не выяв-
лено достоверных различий при культивировании штаммов на богатой сахарами среде МЭА и трёх других полусинтети-
ческих средах. Только для двух штаммов (LE-BIN 2138 и 4355) была обнаружена высокая целлюлолитическая активность
при культивировании на МЭА. Показано отсутствие активности ферментов лигнолитического комплекса при культиви-
ровании штаммов на новых модифицированных полусинтетических агаризованных средах Malus-M и Pyrus-M.

Ключевые слова: ксилотрофные грибы, фитопатогены, культивирование грибов, лигниназы, скорость роста
мицелия, целлюлазы, чистые культуры.

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**Introduction**

This paper continues the series of publications (Volobuev et al., 2019; Shakhova, Volobuev, 2020 a) devoted to the study of the biological peculiarities of xylotrophic macrofungus *Sarcodontia crocea* (Schwein.) Kotl. (*Polyporales, Basidiomycota*), which is a dangerous phytopathogen in orchard agroecosystems of Eastern and Southern Europe (Szczepkowski, 2010). Living apple trees (*Malus domestica, M. sylvestris, etc.*.) are the preferred hosts for the development of this fungus (Fig. 1). The findings of *S. crocea* are also known on representatives of the genera *Pyrus, Prunus, Acer* (Eriksson et al., 1981; Volobuev, Bondartseva, 2012), as well as some other deciduous trees. Taking into account the available data on the occurrences of *S. crocea* basidiocarps and the peculiarities of its substrate preferences, we carried out this research with the aim of comparative study of growth parameters, cultural and morphological characteristics and biosynthetic potential of *S. crocea* strains under cultivation on agarized media of different composition. In addition to the commercial malt extract agar nutrient medium, we used a semi-synthetic glucose-peptone medium with a standard permanent composition containing all the essential macro- and microelements required to grow the mycelium of basidiomycetes. Furthermore, an attempt was made to model the natural growth conditions of the studied strains using semi-synthetic nutrient media based on water extracts from the wood chips of the main host plants – *Malus domestica* and *Pyrus communis*.

**Materials and Methods**

**Origin of Sarcodontia crocea strains.** Fungal strains were isolated *ex situ* both from basidiocarps and basidiospores on the territory of Belgorod, Oryol and Rostov Regions in 2006–2019 using the traditional methods of solid phase cultivation (Shakhova, Volobuev, 2020b). The identification of strains was performed using the ITS1-5.8S-ITS2 nrDNA analysis (Shakhova, Volobuev, 2020a) and based on the cultural and morphological parameters described by J. A. Stalpers (1978). The studied dikaryotic strains of *S. crocea* are maintained in the Komarov Botanical Institute Basidiomycetes Culture Collection (LE-BIN) (Belova et al., 2005).

**Measurement of vegetative mycelium growth rate.** To obtain an inoculum, *S. crocea* strains were grown on a standard medium with malt extract (*MEA; «Conda» 4% m/V*) and agar (*«Difco» 2% m/V*) on Petri dishes (90 mm diam.) and incubated at 25°C in the dark for 14 days.

The linear growth rate of *S. crocea* strains was investigated in four different nutrient media: the «rich» MEA medium, the semi-synthetic agarized (2%) media – glucose-peptone medium (GPA) and two modified «poor» media, referred to as Malus-M and Pyrus-M in this experiment. The content of GPA medium (g/l) was following: glucose – 5.0, peptone – 1.5, KH₂PO₄ – 0.6, K₂HPO₄ – 0.4, ZnSO₄ – 0.001, FeSO₄ – 0.0005, MnSO₄ – 0.05, MgSO₄ – 0.5; pH 5.8. The compositions of Malus-M and Pyrus-M media were identical to GPA, with the exception that the carbon source was the water extracts of commercial wood chips («PALISAD Camp-ings») of *Malus domestica* and *Pyrus communis* respectively.

The water extracts of wood were prepared as follows: 100 g of wood chips were placed in conical flasks (1 l), 500 ml of distilled water was added and sterilised at 1 atm for 1 hour, then kept at 25°C for 24 hours. The resulting liquid was filtered through a nylon fabric and pH was increased to 5.8 using 5% KOH. 2% agar was added to the obtained water extraction from the wood and sterilised again in an autoclave under the regime described above (1 atm for 1 hour). The final concentration of carbon in Malus-M and Pyrus-M corresponded to the carbon concentration in GPA.

The fungal strains were cultivated in mycelial blocks (7 mm diam.), placing them on the nutrient medium in the centre of a Petri dish (90 mm diam.) with a mycelial layer downwards. The growth rate was determined over a period of 28 days, measuring the diameter of the colony (in mm) in two mutually perpendicular planes every two days starting from the third day until the Petri dish was completely overgrown.
Fig. 1. Basidiocarp of *Sarcodontia crocea* on living tree of *Malus domestica*, Belgorod Region, Korochansky district, vicinity of Popovka village, 2019. Photo: S. V. Volobuev.

Рис. 1. Плодовое тело *Sarcodontia crocea* на живом дереве яблони, Белгородская область, Корочанский р-н, окрестности с. Поповка, 2019 г. Фото: С. В. Волобуев.
Assessment of enzymatic activity. The activity of lignocellulolytic complex enzymes was studied using the rapid screening method (Shakhova, Volobuev, 2020b). The strains were grown on agarized MEA, GPA, Malus-M and Pyrus-M media in a thermostat at 25 ºC for 2 weeks. Cultivation of strains was carried out with mycelial blocks 7 mm diam. cut from the edge zone of the actively growing colony, by placing them in the centre of a Petri dish with a mycelial layer upwards. The qualitative activity of oxidative and cellulolytic enzymes in the studied strains was determined by the application method, as described in Shakhova and Volobuev (2020 a).

The statistical analysis of the results obtained was performed using the Origin 7.5 and Microsoft Excel software packages.

Results and discussion

The obtained values of S. crocea growth rates on four different nutrient media are presented in Fig. 2. It has been shown that all media were suitable for the growth of the strains studied, but the growth rate and macromorphology of colonies differed significantly between media. As indicated in Fig. 2, the growth rate of S. crocea cultivated on a sugar-rich MEA medium was significantly higher than on semi-synthetic media. The strain S. crocea LE-BIN 4378 had the lowest growth rate on all the media studied: by the 14th day, colony diameters were 42.5, 21.0, 23.3 and 26.0 mm on MEA, GPA, Malus-M and Pyrus-M media, respectively.

It should be noted that the lowest growth rate for most strains was found on sugar-poor Pyrus-M medium. The complete overgrowth of Petri dishes (90 mm diam.) in S. crocea strains occurred after 30–40 days of cultivation on this medium, while in LE-BIN 2138 and 4343 the growth of the colonies stopped by the 17th and the 21st days (Fig. 2). The exceptions were strains LE-BIN 4367 and 4378, whose growth rate on Pyrus-M was similar to that on Malus-M (Fig. 2). The cultivation of S. crocea on Malus-M was also characterized by lower growth parameters compared to «rich» MEA and semi-synthetic GPA. The complete overgrowth of Petri dishes in most of the strains studied came after 25–30 days of cultivation on Malus-M. However, the growth rate of LE-BIN 4382 on Malus-M was higher than on other semi-synthetic media (Fig. 2).

The significant variability in the macromorphology of colonies for the studied strains was observed when S. crocea was grown on nutrient media of different composition. All the strains studied, when cultured on semi-synthetic media, were characterized by changes in the appearance of colonies compared with the results of cultivation on MEA. In particular, the loss of zonality and distinct air mycelium in the colonies of the strains studied was registered. In addition, when strains were cultivated on semi-synthetic nutrient media, the mycelial mat became sparser and submerged (Fig. 3). The cultivation of strains on semi-synthetic media of Malus-M and Pyrus-M was also accompanied by a change in the reverse towards darkening (Fig. 3, C, D).

The next stage of the research included a comparative study of the biosynthetic potential of S. crocea strains during growth in «rich» MEA as well as media containing water extracts from Malus domestica and Pyrus communus wood chips. It has been shown that the strains studied on all media were characterized by the presence of cellulolytic enzyme activity, which varies depending on the composition of the media (Fig. 4).

The strain LE-BIN 2138 exhibited high enzymatic activity on MEA and GPA, but the cellulolytic activity was significantly reduced when the strain was cultivated on media containing water extracts from Malus domestica and Pyrus communus wood chips (by 34 and 36%, respectively). The strain LE-BIN 4355 also had quite high cellulolytic activity on MEA and GPA and showed a decrease in the activity on Malus-M and Pyrus-M (by 26% and 34% respectively). It is noteworthy that only LE-BIN 2138 and 4355 had high cellulolytic activity when cultivated on a sugar-rich MEA. The rest of the strains either showed no increase in enzymatic activity when grown in MEA (LE-BIN 4365) or were characterized by a decrease in the cellulolytic activity compared to semi-synthetic media (Fig. 4). For example, strains LE-BIN 4342, 4343, 4346, 4367 and 4382 demonstrated a 7–51% lower cellulolytic activity on MEA than in these strains on GPA. The strain LE-BIN 4378 did not reveal the cellulolytic activity when cultivated on MEA (Fig. 4).
Fig. 2. The linear growth rate of *Sarcodontia crocea* LE-BIN strains on MEA, GPA, Malus-M and Pyrus-M nutrient media

Рис. 2. Линейная скорость роста штаммов *Sarcodontia crocea* LE-BIN на питательных средах MEA, GPA, Malus-M и Pyrus-M
The result can be explained by the fact that cellulases, being inducible enzymes (Manavalan et al., 2011; Coradetti et al., 2012), are synthesized only in the presence of the relevant substrate (cellulose) and inhibited by the end products (long- and short-chain oligosaccharides) (Woodward, 1991). Transcription of cellulase genes is known to be suppressed at high concentrations of glucose (Adav et al., 2012; Zang et al., 2018). It is likely that the absence of activity observed during the cultivation of strain LE-BIN 4378 in a sugar-rich MEA medium may be due to conservative and divergent peculiarities associated with the regulation of genes involved in the biosynthesis of cellulolytic complex enzymes in this strain.
is required for induction and secretion of cellulase and hemicellulase genes in basidiomycetes. Different species of wood-decaying fungi synthesize both individual enzymes of the lignocellulolytic complex and multi-enzymatic combinations. Xylotrophic fungi belonging to the same ecological group and having common substrate preferences tend to have a similar composition of enzymes. But the activity level of extracellular enzymes has a significant strain and species variability (Fernandes et al., 2012). The cultivation of LE-BIN 4343 and 4365 on Pyrus-M resulted in a significant increase in the cellulolytic activity (by 25 and 42%). However, when these strains were grown on Malus-M, there was a reduction in enzymatic activity of 44% and 11% respectively (Fig. 4).

The results of the analysis of oxidative enzyme activity are presented in Fig. 5. It was found that all the strains studied had oxidative activity only on a rich MEA and semi-synthetic GPA. Nevertheless, the activity of lignolytic complex enzymes in strains grown on GPA was 25–36% lower compared to MEA. All strains of S. crocea involved in the study demonstrated no oxidative activity when cultivated on Malus-M and Pyrus-M (Fig. 5). The absence of this activity in the studied strains, which is determined by the enzymes of the lignolytic complex, may be related to the use of wood extracts as a nutrient medium. Previously, A. Piscitelli and co-authors concluded that a low rate of oxidative enzymes can be obtained from the growing of fungi on wood (Piscitelli et al., 2011).

An additional reason for the lack of oxidative enzyme activity in the studied strains on Malus-M and Pyrus-M is probably the insufficient quantity of carbon in them. Carbon sources in the medium play an important role in the production of oxidative enzymes, as they can promote mycelium growth and induce the gene transcription of the laccase – one of the lignolytic complex enzymes (Rivera-Hoyos et al., 2013). At the same time, the expression of genes responsible for the production of lignolytic enzymes in Phanerochaete chrysosporium was only triggered when carbon-based nutrients were exhausted (Wang et al., 2019). The initial concentration of the carbon source is known to be a crucial factor in the synthesis of certain lignolytic complex enzymes. A. Tavares and co-authors showed that the cultivation of Trametes versicolor in a nutrient medium with a glucose source concentration (11 g/l) resulted in a maximum laccase production (11403 U/l) (Tavares et al., 2006). It has been noted that the presence in the nutrient medium of rapidly biodegradable substrates such as glucose, mannitol and cellobiose increases the laccase activity, as opposed to slowly degradable substrates (cellulose or lactose) (Rivera-Hoyos et al., 2013). The results of the element analysis of the fruit trees extracts used in this study indicated that the main
components of water-soluble substances from *Malus domestica* and *Pyrus communis* wood chips were carbohydrates, proteins and inorganic salts (unpublished data). It is possible that during the water extraction, some of the natural inducers contained in the fruit tree chips did not flow into Malus-M and Pyrus-M. Some of the lignocellulose residues are known to contain natural inducers that enhance the production of oxidoreductases. W. Qiu and co-authors showed that the solid phase cultivation of *Funalia trogii* in a nutrient medium prepared using the root of *Pueraria montana* var. *lobata* (Fabaceae) (in which flavonoids are the main phenolic compounds) increased the laccase production (Qiu et al., 2014).

In addition to the reasons discussed above, the bioecological features of *S. crocea* are an important factor affecting the absence of oxidoreductase activity when cultivated on Malus-M and Pyrus-M. This species refers to xylotrophic phytopathogenic fungi, which tend to develop on living trunks and thick branches, causing rotting of the wood and causing a death of the tree. Besides the specialisation in development on certain host species (*S. crocea* prefers the wood of *Malus* spp. (Eriksson et al., 1981)), this group of fungi is characterized by being associated with living plants, remaining vitality on dead plant remnants only for a short time (Szczerpkowski, 2010; Shakhova, Volobuev, 2020a). Apparently, chips or nutrient media based on water extracts from the chips of seed fruit trees were treated by *S. crocea* strains as an organic matter of a dead plant, as evidenced by low growth rate, depressed mycelial mat and partial loss of the activity of lignolytic complex enzymes. At the same time, *S. crocea* strains, when cultivated in a rich (malt extract-based) organic MEA, were able to actively grow and produce ligninases and cellulases.

Thus, the nutrient media used in the study proved suitable for the growth of *S. crocea* strains, but the growth rate and macromorphology of fungal colonies differed between media. It has been established that the ability of *S. crocea* to produce lignocellulolytic complex enzymes depends on the composition of the nutrient medium. The cellulolytic activity has been shown on all the agarized media we used, and the content of the media was crucial for the production of oxidative enzymes. The experimental data obtained allowed us to improve the knowledge on the biology of the xylotrophic basidial fungus *S. crocea*.

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