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STABILITY OF BASIDIOMYCETE STRAINS WHEN PRESERVED IN A COLLECTION

S. Miros, Ph.D., Associate Professor
V. Koocherov, research fellow
S. Bilokon, Ph.D., Associate Professor
A. Sechnyak, Ph.D., Associate Professor
Department of Genetics and Molecular Biology
Odessa I.I.Mechnikov National University
Dvoryanskaya str., 2, Odessa, 65082, Ukraine

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Correspondence:

A. Sechnyak
E-mail: sechnyak@ukr.net

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Abstract. Basidial macromycetes can be a material used to develop new biotechnologies, medical preparations, components of dietary nutrition. That is why fungal strains in collections should be kept and identified at the highest level. An important parameter is the stability of isolated and described collection strains of basidiomycetes. Today, it is one of the key problems of long-term preservation of pure cultures in collections. For Odessa I.I.Mechnikov National University's collection of basidiomycetes (preserved by replanting the colonies periodically), the problem of strain stability has not been studied yet. The purpose of this research is to study the stability of this collection by such parameters as the growth rate of mushroom colonies and the electrophoretic spectra of carboxylesterases, with different periods of preservation of the cultures on solid wort agar. The three age groups of strains tested in this research were preserved for 1, 2, and 3 years, respectively, on a wort agar medium at 4°C. The radial growth rate of their vegetative mycelium and the spectra of multiple molecular forms of carboxylesterases have been determined by vertical electrophoresis in 7% polyacrylamide gel. It has been established that the stability of the radial growth rate of the vegetative mycelia of *A. auricula-judae*, *F. velutipes*, and *G. lucidum* after different preservation periods is high according to the values of coefficients of variation. The expressivity of molecular forms of carboxylesterase, though, was quite varied. Partially conservative molecular forms have been detected in some age groups of strains, and in some individual strains, too. So, the growth rate of colonies is a stable parameter, and the molecular forms of carboxylesterases of strains of different age are variable.

Keywords: stability, basidiomycetes, growth coefficient, molecular forms of carboxylesterases.

Introduction. Formulation of the problem

Nowadays, ex-situ collections of fungi are an important resource to solve scientific and practical problems. Mushrooms are widely used in dietary nutrition, they are a valuable material to develop new biotechnologies, to produce medicines, etc., and they are widely used as technological components in food production [1,2]. So, it is important to solve the problem of finding a reliable method to preserve pure fungal cultures.

Analysis of recent research and publications

Now, there are two groups of preservation methods for pure fungal cultures: the modern and the traditional ones. The group of modern methods includes lyophilisation and low-temperature conservation (cryopreservation) [3]. However, for most basidiomycetes, the lyophilisation method is unacceptable [4]. The method of convective air-drying has recently been developed as an alternative to lyophilisation [5].

Depending on the preservation technique used, basidiomycetes can decrease in productivity, lose morphological characteristics and pharmacological activity [6]. Singh et al. [7] observed the viability of most basidiomycete strains after cryopreservation in liquid nitrogen. Instead, Colauto et al. [8] observed that 8 samples out of 12 had lost their viability after four years of cryopreservation at –70°C. Eichlerová et al. [9] found that during storage of fungal cultures in liquid nitrogen, the laccase activity decreased by 21–44%. It is believed that crystallisation of water outside the cell increases the osmotic pressure inside the cell and impairs the activity of enzymes. It has also been found that after freezing at –80°C, the viability is recovered faster than after freezing in liquid nitrogen [10]. Different kinds of cryoprotectants are widely used in cryopreservation. Homolka's perlite protocol and its modifications are very popular. In particular, a mixture of 5% DMSO and 10% trehalose

is a very effective cryoprotectant that can be used to cultivate a lot of cryosensitive strains [11].

Cryopreservation of basidiomycetes by vitrification, or encapsulation, has been reported [12]. The benefits of encapsulation are the reduction of water content in the cells, which reduces the risk of ice damage, and the ease of manipulating the cells [13].

Methods of revitalisation play an important role in cryopreservation. It has been found that, after five years of cryopreservation, a semi-solid growth medium increased the revivability of the *Agaricus subrufescens* mycelium [14]. To revive long-stored cultures more successfully, low-level laser radiation was effectively used to stimulate growth of macromycetes and intensify the technological stages of their cultivation [15].

Traditional storage methods (periodic replanting, preservation under mineral oil, under water, in soil) are affordable and easy to use in the laboratory, but less effective in the long run [3]. At the same time, methods of subculturing have not only methodological, but systematic features as well. The acceptable efficacy of different methods of subculturing dikaryotic and monokaryotic cultures of the pathogenic fungus *Ganoderma boninense* was revealed. After the culture-carrying agar was immersed in mineral oil or water, the viability of the culture samples ranged 50 to 80% [16].

The quality of culture preservation can be improved by using microelements. It has been found that using organic and inorganic selenium compounds increases the preservation time of mycelial cultures at 4°C to as long as 18–24 months. With this method used, the viability and biochemical characteristics of the cultures, the parameters of their growth (its rate and specific character, cytoplasmic basophilia, enzymatic activity, ability to form basidiomas) are retained at a sufficient level [17].

When macromycetes are preserved in a collection, one of their important characteristics is the stability of their basic parameters. When fungal strains are periodically replanted, morphological and physiological changes occur quite frequently: the reports of mitotic recombination in laboratory mushroom cultures are commonly known [18,19]. Besides, there are widely known facts of long periodic replants that ended in karyotypic changes in diploid strains *Saccharomyces cerevisiae* [20] and strains *Magnaporthe grisea* [21]. However, this does not always lead to noticeable changes in morphological and physiological characteristics. When *Ganoderma lucidum* (NBRC 8346) was subcultured 30 times every 45 days, analysis of single nucleotide polymorphism (SNP) in 60 genes before and after the 4 years of preservation detected SNP in 18 genes, and 14 of them were in exons. Non-synonymous coding SNP were found in two genes that code for mevalonate pathway enzymes and in five genes that code for lignin degradation enzymes [22]. So, the preservation of fungal strains at the proper level of genotypic variability is a most important task.

Purpose and objectives of the study. Before, there has been almost no research on the variability of medicinal basidiomycete strains from Odessa I.I.Mechnikov National University's collection (the ONU collection) that were preserved by the traditional method. That is why the purpose of this work was to study the variability of these strains during long-term preservation on wort agar at 4°C. To achieve the purpose, the following objectives have been solved:

1. to study the growth rate of fungal colonies after 1, 2, and 3 years of preservation on thick wort agar at 4°C;
2. to study the electrophoretic spectra of carboxylesterases of fungi after 1, 2, and 3 years' preservation on thick wort agar at 4 °C.

Research materials and methods

The research used strains of the medicinal basidiomycetes, of three age groups of preservation (1, 2, and 3 years), from the ONU collection:

1. *Auricularia auricula-judae* (Bull.) Quél. (Jew's ear) – the strain ONU F201 (Vietnam, Hanoi, 2008);
2. *Flammulina velutipes* (Curtis) Singer (winter mushroom) – the strains ONU F302 yellow (Vietnam, Hanoi, 2014) and F303 (Vietnam, Hanoi, 2014);
3. *Ganoderma lucidum* (Curtis) P. Karst. (Reishi mushroom) – the strains ONU F101 (Vietnam, Hanoi, 2008) and F101a (Vietnam, Hanoi, 2014).

These samples belong to the class *Agaricomycetes*, but are of different orders and families. The Jew's ear belongs to the family *Auriccularіaceae* Fr. Ex Lidau, 1897; the winter mushroom to the family *Phisalacriaceae* Corner, 1970; the Reishi mushroom to the family *Polyporaceae* Cordfs. 1839. Therefore, all the samples are phylogenetically distant. The collection samples were preserved on a wort agar medium at 4°C for 1, 2, and 3 years.

The stability of the samples was evaluated by the variability of the radial growth rate and the spectra of multiple molecular forms (MMF) of carboxylesterases in the vegetative mycelia of the cultures.

To determine the radial growth rate, the fungal cultures aged 1, 2, and 3 years were inoculated on Petri dishes with wort agar (pH 5.8±0.2) and cultured at 22°C until the substrate surface was completely overgrown.

After that, an agar block with a vegetative mycelium (1 cm in diameter) was cut out and placed in the centre of the surface in a Petri dish with a new growth medium. Each collection strain was thus inoculated in triplicate.

On the 10^{th} day of culturing, the colonies of mushroom strains were measured. The modified growth coefficient (GCj) was calculated by the formula [23,24]:

$$GCj = \frac{dhgj}{t},$$

where *d* is the diameter of the colony, mm; *h* is the height of the colony, mm; *g* is the colony density in

points (estimated on a three-point scale: 1 – cobweb-like, 2 – medium, 3 – dense); *j* – homogeneity of the colony in points (estimated on a four-point scale: 1 – the mycelium is heterogeneous, with islands of dense or aerial mycelium, 2 – an inhomogeneous colony, the mycelium is morphologically different only in a separate zone, 3 – the presence of morphologically different mycelium is insignificant, 4 – the mycelium is homogeneous); *t* is the age of the colony, days.

The statistical data were processed according to [25] using the non-parametric Wilcoxon test to compare the samples of values of the colonies' diameter and the modified growth coefficients for each year.

The variability of MMF carboxylesterases was studied by vertical electrophoresis in 7% polyacrylamide gel [26]. The extracts were made from the vegetative mycelium of mushroom strains of different ages. As the extractant, 0.1M glycine-NaOH buffer (pH 9.0) with 1% Triton X-100 was used. The prepared samples were centrifuged in the cold (+4°C) at 10,000 g for 15 minutes, then frozen and thawed [27].

The extracts were subjected to electrophoretic separation under alkaline conditions in a Tris-glycine buffer system (pH 8.9). The 10 µl samples were added to slots, with 5 ml of the 60% sucrose solution and 0.01% bromophenol blue that was used as the leading colour marker. Immediately after the application of the samples, electric current of 5 mA was applied to the starting surface of the gel (for 20 minutes), and then 20 mA. At room temperature, under these conditions, the electrophoresis took 3 hours. As soon as the leading dye reached the finish level, the process was stopped, the gel blocks were released, repeatedly washed with distilled water to neutralise the internal buffer, and used to detect carboxylesterases [14].

The washed gel blocks were incubated for 20 minutes at t=25±0.2ºC in 0.1 M Tris-glycine buffer (pH 7.4) containing diazonium and 25 mg of β-naphthyl propionate used as a substrate [28]. Boiling distilled water was poured onto the plates to inactivate enzymes, thus stopping the hydrolysis of the substrates by esterases. The gel blocks were then repeatedly washed with distilled water and scanned wet in high resolution. The generated scans were then measured densitometrically using the licensed computer program Anais (2004).

Results of the research and their discussion

The growth rate on a standardised complex medium, an example of which is wort agar, is one of the most stable characteristics of strains in a culture. This parameter can be used to evaluate taxonomically different fungi, to compare species of different growth rate or cultures of different age, which is important when selecting fast-growing strains [11].

Using the modified growth coefficient, we have examined the genotypic stability of the collection strains at the level of its manifestation in the phenotype. In particular, modified growth coefficients for collection strains of different age have been established (Table 1).

The comparison of pairs of samples (that were of different age and belonged to the same strain) by the criterion "modified growth coefficient," using the non-parametric Wilcoxon test, has shown that the results can be considered statistically reliable ($U_{factual} > U_{table}$ at $p=0.05$). Therefore, samples of different age belong to the same general population or different general populations with the same parameters, i.e. they have similar growth characteristics.

According to the criterion "modified growth coefficient," coefficients of variation did not exceed 10%. Therefore, variability by the values of this parameter can be considered insignificant, and the genotypic stability of the collection strains after one year, two, and three years of preservation quite high.

Table 1 – Growth rates of some strains from the basidiomycete collection in three-year dynamics

Species	Strain	Age, years	Parameter					
			Colony diameter ($d \pm s_d$, *mm*)	Colony height (*h*, *mm*)	Colony density, (*g*, *point*)	Colony homogeneity (*j*, *point*)	Modified growth coefficient ($GCj \pm s_{PKj}$)	Coefficient of variation (C_v, *%*)
G. lucidum	F101	1	92.67±1.15	2	1	2	39.1±0.46	1.25
		2	88.00±1.00	2	1	1	32.4±0.20	1.14
		3	85.00±2.65	2	1	1	37.1±0.53	3.11
	F101a	1	94.00±1.73	1	1	1	52.3±0.23	1.84
		2	93.00±1.73	1.5	2	2	56.0±1.15	1.86
		3	82.00±2.65	1.5	2	2	55.8±1.76	3.23
auricula	F201	1	85.67±2.52	2	2	1	39.6±1.01	2.94
		2	89.67±2.52	2	2	1	35.7±1.01	2.81
		3	94.00±2.65	2	2	1	37.8±1.06	2.81
F. velutipes	F302	1	94.00±2.65	4	1	2	76.5±2.12	2.81
		2	93.33±3.06	3	1	2	65.4±1.83	3.27
		3	94.33±0.58	2	1	2	69.5±0.27	0.61
	F303	1	81.67±3.79	3	2	3	125.7±7.57	4.64
		2	94.00±3.46	4	1	3	112.2±4.16	3.69
		3	85.00±2.65	3	1	3	117.9±2.38	3.11

Carboxylesterases, which function as lipases in fungi, are quite indicative when modification and genotypic variability is investigated at the enzymatic level [24,27]. The procedure optimised for mushrooms allows determining isoforms that are clearly distinguished on electrophoretic spectra, which can give an idea of how the biochemical activity of mycelium changes in the course of preservation. The electrophoretic study of the vegetative mycelium of the collection strains allowed determining 9 MMF of carboxylesterases, their Rf (coefficient of mobility), and expressivity. The latter was expressed in relative optical density units (ΔD_o). The results are shown in Fig. 1 and in Table 2.

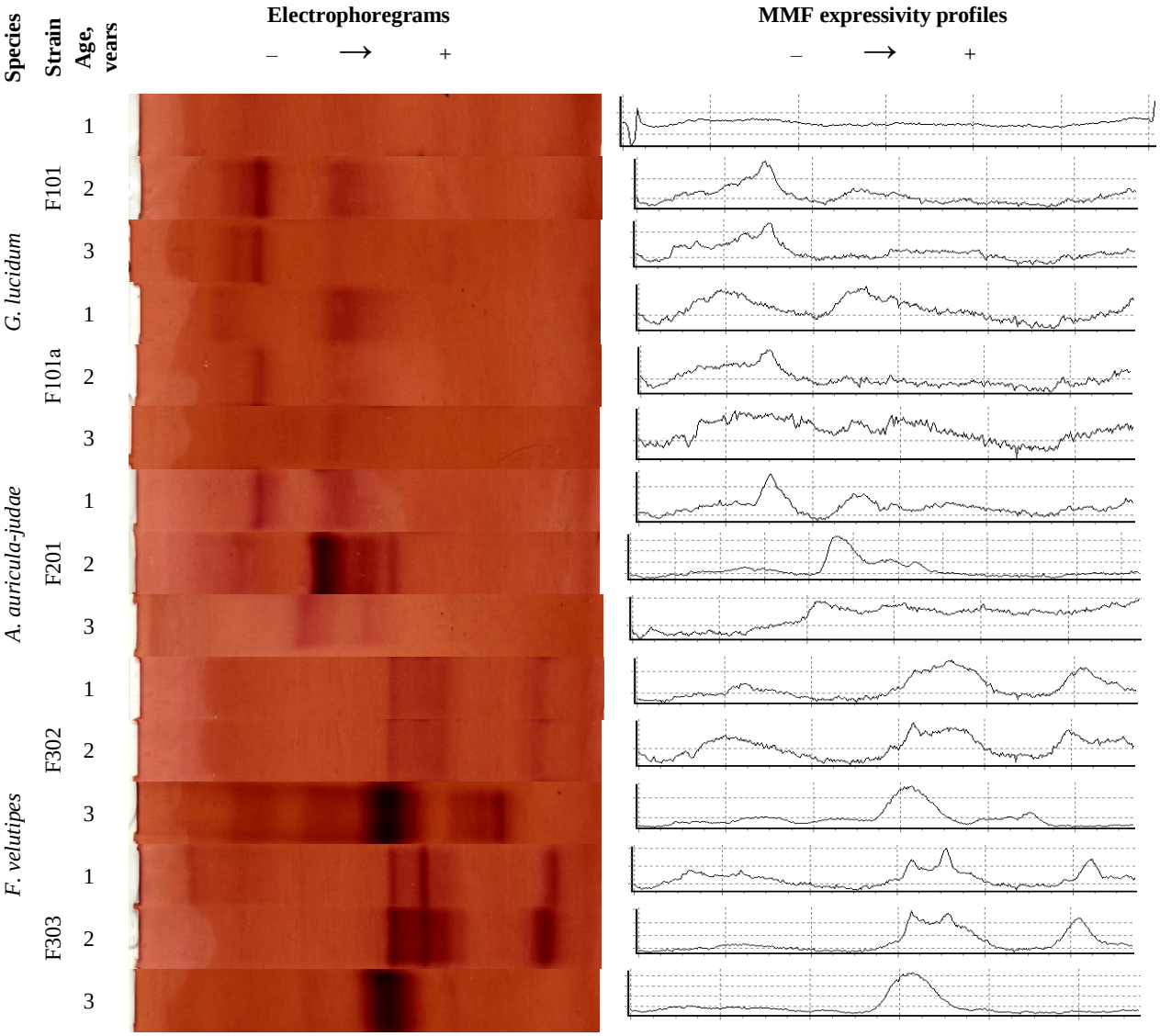

Fig. 1. Electrophoregrams of the vegetative mycelium extracts and graphs of the expressivity of MMF of carboxylesterases

For most MMF carboxylesterases, considerable variability was observed during the preservation of basidiomycetes strains. The spectra and the expressivity characteristics of MMF enzymes changed with the increase of the samples' preservation period (Table 2).

Carboxylesterases common to all the species and strains under study have not been detected. At the same time, forms with Rf=0.55 have been detected in all species and strains that are three years old, and those with Rf=0.21 have been found in the strains F101, F201, F302, and F303 that are two years of age and represent all the three species studied.

Molecular forms have been detected present in certain strains irrespective of the preservation age: in F101a – forms with Rf=0.43; in F303 – forms with Rf=0.55; in F302 – forms with Rf=0.87. The most common among the samples were MMFs with Rf=0.43 and 0.55. The form most intensively expressed is the one with Rf=0.55, in particular for the strains F201, F302, and F303. Forms with Rf=0.43 and 0.87 are also expressed quite intensively. A minor isoform of carboxylesterases was the form with Rf=0.36.

Table 2 – Expressivity of MMF of carboxylesterases in strains of the collection basidiomycete samples

Species	Strain	Age, years	Rf								
			0.10	**0.17**	**0.21**	**0.26**	**0.36**	**0.43**	**0.55**	**0.63**	**0.87**
G. lucidum	F101	1	-	-	-	-	-	-	-	-	-
		2	-	-	0.714	-	-	0.339	-	-	-
		3	-	-	0.489	0.582	-	-	0.329	-	-
	F101a	1	-	0.410	-	-	-	0.426	-	-	-
		2	-	-	-	0.464	-	0.257	-	-	-
		3	-	0.381	-	-	-	0.337	0.362	-	0.333
A. auricula-judae	F201	1	-	-	-	0.510	-	0.331	-	0.270	-
		2	-	-	0.375	-	-	1.070	0.540	-	-
		3	-	-	-	-	0.216	-	0.181	-	-
F. velutipes	F302	1	-	-	0.358	-	-	-	-	0.624	0.536
		2	-	-	0.331	-	-	0.405	-	-	0.358
		3	-	-	-	0.490	-	0.445	1.260	-	0.623
	F303	1	-	-	-	-	-	-	0.960	0.927	0.817
		2	0.441	-	0.417	-	-	-	0.552	0.691	0.585
		3	-	-	-	-	-	-	1.080	-	-
Σ			0.441	0.791	2.684	2.046	0.216	3.610	5.264	2.512	3.252

Note: the values in the table show the expressivity of the MMF of enzymes which is expressed in relative optical density units (ΔDo) of the corresponding enzyme-containing fractions in the gel block.

The samples of strains belonging to the same species (F101 and F101a to *G. lucidum*, F302 and F303 to *F. velutipes*) had three and four common forms, respectively.

This fact may be due to the polyallelism and polylocus of carboxylesterases, as well as to the existence of post-translational modifications under the conditions of long-term preservation. The effects of the ontogenetic mechanisms of activation/inhibition of certain loci cannot be excluded either.

To date, there is not enough information on the genetic control of carboxylesterases in the species considered. There are only isolated data for certain types of macromycetes. For example, in *Pleurotus ostreatus* PC15, 20 genes that encode carboxylesterases have been identified [29].

As a result of our study, it has been established that during three years of preservation, mushroom cultures show phenotypic stability by the growth rate criterion, whereas the expressivity of MMF of carboxylesterases is quite variable. However, partially conservative forms have been observed, too. Those with Rf=0.55 have been detected in all three-year-old strains, and the ones with Rf=0.21 in the strains F101, F201, F302, and F303. Also in all the samples of F101a, forms with Rf=0.43 have been found; in all the samples of F303, forms with Rf=0.55, and in all the samples of F302, forms with Rf=0.87. Taking into account that these strains belong to phylogenetically distant species (regardless of whether there are any posttranslational modifications, or whether an ontogenetic programme of changing the activity of certain loci is implemented), it can be expected that these forms have adaptive value for collection cultures.

Thus, during the three-year-long preservation, the collection cultures of basidiomycetes observed showed no changes by their morphological feature, the rate of growth of the vegetative mycelium, but showed variability by the biochemical criteria.

Conclusion

1. The growth rate of the investigated strains did not change during 1, 2, and 3 years of preservation on wort agar.

2. Throughout the preservation period of the collection strains, the electrophoretic spectra of multiple molecular forms of carboxyesterases in mushroom mycelium were changing. Only the forms with Rf=0.43 in the strain F101a, the forms with Rf=0.55 in the strain F303, and the form with Rf=0.87 in the strain F302 remained constant.

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СТАБІЛЬНІСТЬ ШТАМІВ БАЗИДІАЛЬНИХ МАКРОМІЦЕТІВ ПРИ ЗБЕРІГАННІ У КОЛЕКЦІЇ

С.Л. Мірось, кандидат біологічних наук, доцент**,** e-mail: s.miros@onu.edu.ua
В.О. Кучеров, наук. співробітник, e-mail: kooch.victor@gmail.com
С.В. Білоконь, кандидат біологічних наук, доцент, e-mail: s.v.belokon@onu.edu.ua
О.Л. Січняк, кандидат біологічних наук, доцент, e-mail: a.sechnyak@onu.edu.ua
кафедра генетики та молекулярної біології,
Одеський національний університет імені І.І. Мечникова,
вул. Дворянська, 2, Одеса, 65082, Україна

Анотація. Базидіальні макроміцети можуть служити матеріалом для розробки нових біотехнологій, отримання медичних препаратів, бути компонентами дієтичного харчування, тому потребують найбільш високого рівня якості підтримування та ідентифікування штамів у колекціях. Важливим параметром при цьому є стабільність виділених та описаних колекційних штамів базидіоміцетів, яка сьогодні є одною з ключових проблем довготермінового зберігання колекцій чистих культур. Для колекції базидіоміцетів ОНУ імені І.І. Мечникова, яка зберігається методом періодичних пересівів, вона не досліджена. Метою даної роботи є вивчення стабільності зазначеної колекції за швидкістю росту колоній грибів та електрофоретичним спектрам карбоксилестераз при різних строках зберігання культур на твердому сусло-агарі. У роботі використовували штами трьох вікових категорій зберігання на сусло-

агаровому середовищі при температурі 4°С протягом 1, 2 і 3 років. Визначали радіальну швидкість росту їхнього вегетативного міцелію, а також спектри множинних молекулярних форм карбоксилестераз методом вертикального електрофорезу в 7%-м поліакриламідному гелі. Встановлено, що стабільність параметра радіальної швидкості росту вегетативного міцелію *A. auricula-judae, F. velutipes, G. lucidum* різного строку зберігання висока у відповідності із значеннями коефіцієнтів варіації. В той же час, експресія молекулярних форм карбоксилестераз оказалась достатньо мінливою. Спостерігалися і частково консервативні молекулярні форми, котрі були детектовані для окремих вікових груп штамів, а також для окремих штамів. Отже, швидкість росту колоній є стабільним показником, а молекулярні форми карбоксилестераз штамів різного віку варіабельні.

Ключові слова: стабільність; базидіоміцети; ростовий коефіцієнт; молекулярні форми карбоксилестераз.

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