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EVALUATION OF WILD STRAINS OF *PLEUROTUS OSTREATUS* ISOLATED FROM SPONTANEOUS MYCOBIOTA BY MYCELIUM GROWTH CHARACTERISTICS *IN VITRO*

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Abstract
Specimens of Pleurotus ostreatus mushrooms from spontaneous mycobiota were collected from different regions of Romania, in order to obtain mycelial isolates, preserve and use them for the development of the cultivated assortment. The mycelia of the PoBr and Po2T strains, obtained from a first collection series, were clonally multiplied and cultured in vitro to highlight the morphophysiological and cultural characteristics of the colonies, compared to two commercial strains of P. ostreatus. The wild isolates have developed on three variants of agarized media – with potato extract (PDA), with malt extract (MEA) and with compost extract (MECA) – viable, robust mycelial colonies, with morphology similar to that of commercial strains but also with their own peculiarities. The growth rate of wild mycelia was lower than in commercial ones. A similar behavior of the new isolates was highlighted during the preparation of the seeding mycelium/spawn grown on wheat grains and, subsequently, during the colonization period, on the lignocellulosic substrate prepared in two variants for the fruiting tests.

Keywords: lignocellulosic substrate, mycelium, Pleurotus ostreatus, wild strain.

1. INTRODUCTION

Macrofungi from various terrestrial ecosystems represent an important genetic resource that must be scientifically studied, evaluated, and preserved in order to be used anytime for the development of the mushroom production industry and a wide range of biotechnological applications. Many of them have exceptional nutritional and/or therapeutic value, which is why they are increasingly used for the production of functional foods, dietary supplements, and traditional medicines (Valverde et al., 2015; Pohleven et al., 2016; Chang and Wasser, 2017; Dupont et al., 2017; Golak-Siwulska et al., 2018; Chang and Buswell, 2021). Other major biotechnological applications of macrofungi include those in the field of mycoremediation of pollutants, recycling of agro-industrial waste, production of enzymes and organic bio-fertilizers, and production of bioethanol (Lu et al., 2020; El-Ramady et al., 2022; Niego et al., 2023).

Romania benefits from a very rich, diverse, and still healthy spontaneous mycobiota, present in all regions within forest ecosystems, pastures, meadows, plains, etc. Macromycetes – over 1600 species, of which 400 are edible – are generously represented here by valuable genera and species (Sălăgeanu and Sălăgeanu, 1985; Șesan and Tănase, 2004). The isolation, preservation, and evaluation of their mycelia for growth and fruiting capacity represent the first steps towards their introduction into cultivation and/or use in other biotechnological applications.

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The classic taxonomy of fungi exclusively uses morphological, cultural, and biochemical characteristics. Their discriminatory capacity is not perfect, as it has certain limitations. Nevertheless, these criteria are still widely used as they always constitute valuable complementary elements, guiding the determination of a certain systematic classification at the species/genus level, ultimately based on molecular genetics techniques. The evaluation of the biological characteristics (morphological, physiological, genetic, etc.) of the mycelium of wild isolates collected from nature helps in their taxonomic identification during biotechnological cultivation, in the absence of fruiting body development. Characteristics such as growth rate, type and morphology of the filamentous colony, color and density of the mycelial network, presence of concentric growth rings, droplets of exudate, and clamp connections, as well as the potential appearance of primordia (teleomorphs), etc., are considered to be significant from a taxonomic point of view and assist in the correct identification of fungal cultures of the species studied during their monitoring in the vegetative growth stage. (Badalyan and Borhani, 2019; Mykchaylova et al., 2019).
Several *in vitro* studies regarding the isolation and characterization of some species of lignicolous macromycetes collected from our forests have highlighted a great diversity of morphological characteristics and fungal colony growth (Bălăieș and Tănase, 2012; Petre and Tănase, 2013; Popa et al., 2014). In this paper, we aimed to evaluate according to the criteria mentioned above the growth behavior in various types of nutrient media of two wild strains of *Pleurotus ostreatus* isolated from different geographical areas, the counties of Prahova and Argeș, compared to two commercial strains of the same species.

2. MATERIALS AND METHODS

Specimens of mushroom *Pleurotus ostreatus* from the spontaneous mycobiota of the broadleaf forest ecosystem were collected from two different regions - Prahova and Argeș counties. Dykariotic cultures PoBr and Po2T were obtained by tissue culture method. The wild isolates were clonally multiplied for studying the morphological and growth characteristics of colonies, compared with two commercial strains of *P. ostreatus*, PoM-77 and PoM-421, from the RDIVFG Vidra collection.
Mycelia were grown onto 90 mm Petri dishes (triplicates), using three types of agar media with a pH value of 6.5: malts extract agar (MEA), potato dextrose extract agar (PDA), malt extract + compost extract (1/1) agar (MECA). Incubation was carried out in darkness at 24-26ºC for 12 days. The growth of the mycelium, expressed in mm and mm/day as appropriate, was determined after 7/10/14 days of incubation by measuring the diameter of the mycelial colonies along two perpendicular axes and calculating the average of these for three repetitions/experimental variant. The mycelia were then checked for growth on granular support made from wheat grains distributed in large tubes (250x30 mm) and incubated in the dark at 24-26°C for 14 days.
For the evaluation of mycelial growth on lignocellulosic substrates, three different recipes were tested: 100% cereal straw (V_0), 100% broadleaf sawdust (V_1) and 50% straw + 50% sawdust (V_2), distributed in 250 ml glass bottles. After sterilization, 100 min at 123°C, the bottles were inoculated and then incubated at 24-26ºC in the dark, for 16 days. Observations and measurements were made regarding the morphology and growth of the colonies.

3. RESULTS AND DISCUSSIONS

The morphophysiological and cultural characteristics of the wild isolates of *Pleurotus ostreatus* obtained from spontaneous mycobiota were highlighted by cultivating them on three types of agar

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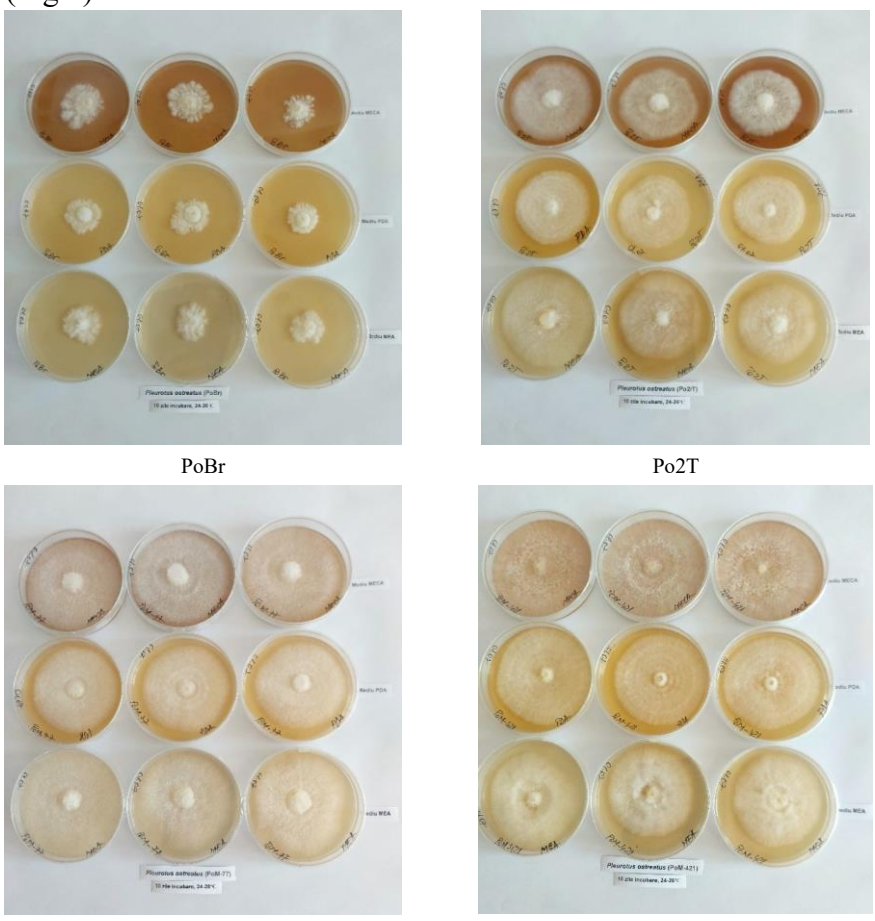
media, on granulated support made from cereal grain and on lignocellulosic substrate identical to that used in mushroom farms.

The two commercial strains of *Pleurotus ostreatus* PoM-77 and PoM-421 displayed vigorous colonies with a uniform appearance and common characteristics, typical of the species on agarized medium variants: white fluffy-cottony mycelium, with medium aerial development, concentric stripes, and small droplets of slightly yellowish exudate. In some colonies, small primordia emerged after 12-14 days of incubation, indicators of the teleomorphic stage. The colonies of these two strains grew faster than those of the wilde isolates, covering the surface of the culture media after 12-14 days of incubation. They did not change the color or consistency of the media they grew on (Fig.1).

The wild strain *Pleurotus ostreatus* PoBr produced mycelial colonies with similar morphology on the three media variants, white and cottony, with medium density and aerial development, featuring a few concentric growth bands of different texture, slightly sectorized and with irregular edges. And the Po2T strain also presented a mycelium with typical characteristics of *Pleurotus ostreatus* - white, uniformly, fluffy-cottony with fine and dense hyphae and a medium aerial development. The colonies show very similar morphology on the three agar media, with well-defined edges and a circular contour (Fig.1).



PoBr Po2T

PoM-77 PoM-421

Figure 1. ***Pleurotus ostreatus - wild strains (PoBr, Po2T) and commercial strains (PoM-77, PoM-421).*** Culture media: MECA - upper; MEA - middle; PDA - lower. (10 days of incubation at 24-26ºC)

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After 10 days of incubation, the highest growth of mycelial colonies was recorded in the commercial strains cultivated on the MECA nutrient medium, with statistically significant results ensured. Thus, the strain PoM-421/MECA grew 80 cm in diameter (8 mm/day), surpassing the control PoM-77/PDA by 11 mm. The strain PoM-77/MECA followed with a growth of 79 mm in diameter (7.9 mm/day), exceeding the control by 10 mm. The lowest increases were displayed by the wild isolated PoBr in all three medium variants, recording statistically very significant negative differences compared to the control PoM-77/PDA: 23.3 mm/PDA (-45.7 mm compared to the control), 28.6 mm/MEA (-40.4 mm compared to the control), and respectively 35 mm/MECA (-34 mm) (table 1).

Table 1. ***The combined influence of strain and agarized nutrient medium on the mycelial growth of four Pleurotus ostreatus strains (10 days of incubation at 24-26ºC)***

No	Strain	Nutrient medium	Mycelium growth		Difference (± mm)	Significance
			Diameter of the colony Mean (mm)	%		
1	*P. ostreatus* PoM-77	PDA	69	100	0	Control
2		MEA	75.6	109.6	+6.6	xxx
3		MECA	79	114.5	+10	xxx
4	*P. ostreatus* PoM-421	PDA	69.6	100.9	+0.6	ns
5		MEA	76	110.1	+7	xxx
6		MECA	80	115.9	+11	xxx
7	*P. ostreatus* PoBr (wild)	PDA	23.3	33.6	-45.7	000
8		MEA	28,6	44.5	-40.4	000
9		MECA	35	50.7	-34	000
10	*P. ostreatus* Po2T (wild)	PDA	58	84.05	-11	000
11		MEA	66	95.6	-3	00
12		MECA	64.6	93.6	-4.4	000
		DL 5% =		1.72		
		DL 1% =		2.34		
		DL 0.1% =		3.15		

The experiment clearly demonstrated the strong influence exerted by the strain on the growth capacity of the mycelium. Thus, the wild isolates PoBr and Po2T showed lower growth on all three types of agar-based nutrient medium compared to the commercial strains PoM-421 and PoM-77, with significant negative differences relative to the control PoM-77/PDA, statistically ensured.

The nature of the nutrient medium has also exerted a very significant influence on the growth of mycelial colonies. The MECA nutrient medium favored higher growths of mycelium of the experimental strains, compared to the MEA and PDA media, in this order. The explanation would be that the malt extract medium enriched with compost extract provides a surplus of nutrients, especially nitrogen, which promotes the growth of mycelium, giving it an additional advantage compared to the other nutrient media used in our experiment.

Furthermore, the growth of the mycelium of the four experimental strains was checked on one of the specific nutrient supports/substrates prepared for the growth and propagation of mycelium intended for spawning - the support made of cereal grains.

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On the granular support (wheat grains), the mycelia of the four experimental isolates exhibited morphological characteristics and growth patterns of the colonies similar to those recorded on agar media.

The growth of mycelium on the granular support distributed in glass tubes was evaluated after 14 days of incubation. The commercial strain *P. ostreatus* PoM-421 recorded the best result among the four experimental strains, namely an average colony growth value of 121 mm (8.6 mm/day), with a very significant difference of +24.4 mm compared to the control PoM-77.

The wild isolates PoBr and Po2T, just like on agar nutrient media, exhibited the slowest growth, showing very significant negative differences compared to the control PoM-77, namely -41.6 mm for the isolate PoBr and -24.3 mm for Po2T (table 2).

Table 2. ***The influence of strain on the growth of Pleurotus ostreatus mycelium on granular substrate (14 days of incubation at 24-26ºC)***

No	Strain	Mycelium growth		Difference (±mm)	Significance
		Mean (mm)	%		
1	*P. ostreatus* PoM-77	96.6	100	0	Control
2	*P. ostreatus* PoM-421	121	125.2	+24.2	xxx
3	*P. ostreatus* PoBr (wild)	55	56.9	-41.6	000
4	*P. ostreatus* Po2T (wild)	72.3	75.7	-24.3	000
DL 5% =				4.09	
DL 1% =				6.19	
DL 0.1% =				9.95	

Table 3. ***The combined influence of the strain and the substrate on the growth of the mycelium in four strains of Pleurotus ostreatus (14 days of incubation at 24-26ºC)***

No	Strain	Substrate	Mycelium growth			Significance
			Mean (mm)	%	Difference (±mm)	
1	*P. ostreatus* PoM-77	V_0	82.3	100	0	Control
2		V_1	115	139.7	32.7	xxx
3		V_2	105.7	128.4	23.4	xxx
4	*P. ostreatus* PoM-421	V_0	108.7	132	26.4	xxx
5		V_1	120	145.8	37.7	xxx
6		V_2	115.3	140	33	xxx
7	*P. ostreatus* PoBr (wild)	V_0	48	58.3	-34.3	000
8		V_1	63	76.5	-19.3	00
9		V_2	59.7	72.5	-22.7	00
10	*P. ostreatus* Po2T (wild)	V_0	66.3	80.5	-16	0
11		V_1	67.3	81.7	-15	0
12		V_2	88.3	107.2	6	ns

* V_0 – 100% straw-based substrate; V_1 – substrate based on 100% broadleaf sawdust; V_2 – substrate based on a mixture of 50% straw + 50% broadleaf sawdust

DL 5% =	12.99
DL 1% =	17.71
DL 0.1% =	22.80

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V_0: 100% straw-based substrate

V_1: substrate based on 100% broadleaf sawdust

V_2: substrate based on a mixture of 50% straw + + 50% broadleaf sawdust

Figure 2. ***The influence of the strain on the growth of Pleurotus ostreatus mycelium on three types of lignocellulosic substrate. (14 days of incubation at 24-26°C)***
Left - center: wild strains PoBr and Po2T. Right - center: commercial strains PoM-77 and PoM-421

For the evaluation of mycelial growth on lignocellulosic substrates, three different recipes were tested, in which the raw material consisted of 100% cereal straw (V_0), 100% broadleaf sawdust (V_1) and 50% straw + 50% sawdust (V_2), distributed in 250 ml glass bottles. Natural nutritional supplements were added to the materials (9%), represented by a mixture of wheat bran, cornmeal, and sunflower seed meal, in equal proportions.

From figure 2 and from the data presented in table 3 regarding the combined influence of the strain and the culture substrate on the growth of the four *Pleurotus ostreatus* mycelia, it appears that, after 14 days of incubation, the highest growth of mycelial colonies was achieved by the commercial

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strains cultivated on substrate variants V_1 (100% sawdust) and V_2 (50% straw + 50% sawdust). The strain-substrate variant PoM-421/V_1 achieved the best result, surpassing the control PoM-77/V_0 by a very significant difference of 37.7 mm. The same strain, grown on the substrate variant V_2 (50% straw + 50% sawdust), ranked second, with a very significant growth increase of 33 mm compared to the control. It was followed by the strain PoM-77 grown on substrate V_1 (100% sawdust), which exceeded the control by a very significant difference of 32.7 mm. The smallest increases were recorded in the wild strain mycelia, across all three culture substrate variants. Unlike commercial strains, wild isolates exhibited much lower growth capacity values compared to the control PoM-77/V_0, particularly PoBr, with very significant differences of -34.3 mm at PoBr/V_0 and distinctly significant differences of -22.7 mm at PoBr/V_2, and -19.3 mm at PoBr/V_1.
The wild strains PoBr and Po2T showed significantly lower growth values on these substrates compared to the commercial strains PoM-421 and the control PoM-77, with the negative differences from the control being very significant This result can be explained by the fact that commercial strains are usually the product of a selection-breeding process, one of its clearly defined objectives being a high mycelium growth rate to rapidly colonize the culture substrate to the detriment of competing and/or pathogenic microorganisms. Furthermore, rapid colonization shortens the incubation period and the culture cycle.

4. CONCLUSIONS

Although they exhibited lower growth values compared to the commercial strains they were tested against in these initial tests, the mycelia of the wild isolates PoBr and Po2T proved to be viable, robust, and with unique characteristics that recommend them for future uses.

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