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Biological features of *Sparassis laminosa* Fr. (Sparassidaceae, Polyporales) and the main aspects of its reproduction in the territory of Hutsulshchyna National Natural Park, Ukraine

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Abstract

Sparassis laminosa Fr. is a valuable edible fungus and is protected in many countries, including Ukraine. The study of this fungus in the laboratory and its reproduction under natural conditions are research priorities to preserve this species. We studied the culture growth *S. laminosa* strain 2211 on nutrient agar media and different plant substrates. Grain-containing substrates were optimal for mycelial growth, the colonization of which occurred from days 10-20 of the experiment. *S. laminosa* strain 2211 can be grown in the natural environment using the *re-situ* technique. However, fruit bodies were observed in only one location (near *Quercus robur* in a sunny area of a temperate deciduous forest, territory of the village Verbovets) at four months after inoculation of the composite substrate containing wheat grain: coniferous sawdust: sunflower seed shells: wheat straw at a ratio of 67%:17%:8%:8%. The obtained fruit bodies were typical of the species morphology, but they had a considerably smaller size. In addition, only substrates that were completely colonized with mycelium were suitable for the application of *S. laminosa* in nature. The use of partially colonized substrates was accompanied by contamination and growth of *Schizophyllum commune* fruit bodies. Monitoring of the periodicity of the fruiting within the fixed locality and the effectiveness of all 15 the substrates used in experiment for the fruiting of *S. laminosae* strain 2211, will be conducted in future studies.

Key words – fruiting body – mycelial growth – plant substrate – *re-situ*

Introduction

The genus *Sparassis* Fr. in the worldwide is represented by 17 species, 27 records (according to Index Fungorum in June 2019; http://www.indexfungorum.org/). The genus *Sparassis* in the territory of Ukraine is represented by three species: *Sparasis laminosa* Fr., *S. crispa* (Wulfen) Fr., and *S. nemecii* Pilát & Veselý (Didukh 2009, Leshan & Pakhomov 2009, Heluta et al. 2016). *Sparassis laminosa* was found in ecotopics of the east of Ukraine and entered in the "List of species that are in a threatened state and subject to protection" (Leshan & Pakhomov 2009). It is a valuable edible, decorative, and phytopathogenic mushroom, causing yellow-brown rot of roots at the base

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of hardwood trees, mainly including oak (*Quercus robur* L.). Individual fruit bodies reach up to 60 cm in diameter and weigh up to 9 kg, appearing in August-September (Vinayev & Gapiyenko 2002). *Sparassis laminosa* is included in the Red Book of Belarus (third category) (Anonymous 2006), to the Red Book of certain regions of the Russian Federation (Anonymous 2011), to the Red List of the Republic of Poland (V status) (Anonymous 1997–2019).

The biological features of *S. laminosa* cultures on various nutrient agar media have been extensively studied by Sukhomlyn (2000a), Lomberg & Solomko (2012) and Tsvyd et al. (2016). Monokaryons of *S. laminosa* show significant variability in growth rate, biomass accumulation, and tree-decay activity (Sukhomlyn 1997). The cumulative capacity for the accumulation of cadmium and manganese in fruit bodies and mycelium grown in culture have been described by Sukhomlyn (2000a). High values of fibrinolytic activity in *S. laminosa* were studied by Sukhomlyn & Aguzhen (1999). However, no studies have examined the possible reproduction of *S. laminosa* under natural conditions, an important phenomenon because of the often unlawful collection of *Sparassis* fruit bodies, which generally hinders the reproduction of mushrooms of this species. In addition, *S. laminosa* is a promising species for cultivation as a high-quality edible fungus (Mudryk et al. 2000). Additionally, *S. laminosa* shows significant antagonistic activity toward the pathogenic species *Heterobasidion annosum* (Fr.) Bref. (Sukhomlyn 2000b).

Therefore, the aim of this work was to study the peculiarities of the growth of *Sparassis laminosa* 2211 on compositions of various plant substrates and the possibility of applying the overgrowth of these substrates to reproduce the fungus under natural conditions using the author's *re-situ* technique (Pasailiuk et al. 2018).

Materials & Methods

Mycelial culture

The pure culture of *Sparassis laminosa* strain 2211 obtained from the Mushroom Culture Collection of the M.G. Kholodny Institute of Botany of the National Academy of Sciences of Ukraine (IBK), #– 1152 in the international database of the World Federation for Culture Collections – WFCC (Bisko et al. 2016, World Data Center for Microorganism 2019).

Culture growth were observed on Potato Dextrose Agar (PDA), Malt Extract Agar (MEA), and Malt Extract Agar supplemented with the sawdust of the white fir *Abies alba* Mill. (MAA), pH 6.0. The final modification was based on the results of Tsvyd et al. (2016), MEA was purchased from Merck (Darmstadt, Germany). To prepare 1 L of PDA, a decoct obtained after 30 minutes of boiling 200 g finely chopped potatoes was supplemented with 10 g of glucose and 20 g of agar, and then the volume was adjusted to 1 L with distilled water. To prepare 1 L of MAA, we used Malt Extract Agar, in which distilled water was substituted with a decoct obtained from the boiling of fir sawdust for 15 minutes (1% solution). All three media were sterilized for 20 minutes at 121ºC and poured into 90-mm Petri dishes.

The mycelium of 14-day cultures grown on MEA was used as the inoculum. Mycelial disks (5 mm in diameter) were cut with a sterile steel tube along the edge (up to 10 mm) of an actively growing colony. Subsequently, they were transferredand incubated at 26±0.1°C. The radial growth rate of the mycelium was calculated according to Lomberg & Solomko (2012). We recorded the time of the beginning of growth (the appearance of mycelium outside the inoculating disc). The radius of the colonies was measured in two perpendicular directions every day until full envelopment of the medium. The average speed of radial growth (Vr, mm/day) was calculated after 3-5 parallel measurements according to the following formula:

$$Vr = (r_1-r_0)/(t_1-t_0),$$

where r_1 - radius of the colony at the end of growth, mm; r_0 - radius of the colony at the beginning of the phase of linear growth, mm; t_1-t_0 - duration of the linear phase of growth, day.

Macro-morphological features of the mycelium were described according to Stalpers (1978). The macromorphological characteristics of the colony included a description of its texture, color, odor of the mycelium, and color of the reversum. For verification of the identity of the fungal colonies obtained in agar culture, after each culture transfer, we deposited one plate in the thermostat until the formation of a teleomorph.

Spawn preparation

The dynamics of the growth of vegetative mycelium of *S. laminosa* was recorded on the following substrates and their combinations (Table 1).

Table 1 Composition of the experimental substrates № 1 – № 15 (ratios of components are indicated in %).

Components of the substrates	**Names of the substrates**														
	1 WgCsSs	**2 WgCsSsWs**	**3 WgPsSsPns**	**4 CsSs**	**5 CsSsWs**	**6 PsSsPns**	**7 OlWg**	**8 OlCsWg**	**9 OlWgWs**	**10 CsOlWgWs**	**11 Sunflower**	**12 SsCs**	**13 Sawdust**	**14 Bamboo**	**15 Wheat**
Wheat grain (Wg)	40	67	50	-	-	-	16.7	9	14.3	16.7	-	-	-	-	100
Conifer sawdust (Cs)	40	17	-	67	50	-	-	45.5	-	33.3	-	9	100	-	-
Sunflower seed shells (Ss)	20	8	12.5	33	25	25	-	-	-	-	100	91	-	-	-
Wheat straw (Ws)	-	8	-	-	25	-	-	-	14.3	16.7	-	-	-	-	-
Pumpkin seeds shells (Ps)	-	-	25	-	-	50	-	-	-	-	-	-	-	-	-
Peanut shells (Pns)	-	-	12.5	-	-	25	-	-	-	-	-	-	-	-	-
Oak forest litter (Ol)	-	-	-	-	-	-	83.3	45.5	71.4	33.3	-	-	-	-	-
Bamboo sticks	-	-	-	-	-	-	-	-	-	-	-	-	-	100	-

A mixture of coniferous chips was obtained during the shredding of healthy silver fir wood (*Abies alba*) and Scot's pine (*Pinus sylvestris* L.), and the size of the wooden particles was 10×10-40×1 mm. The sawdust of coniferous species was obtained from the shredding of healthy wood of fir and common pine; the size of the wooden particles was 5×2×1 mm. Sunflower seed, pumpkin, and peanut shells were pre-dried. The wheat grain was cooked for 25-30 minutes at the ratio of 10 kg of grain per 10 L of water. After drying, the grain was mixed with 12 and 3 g of gypsum and chalk, respectively for 1 kg of grain. Forest litter was collected under the oak (*Quercus robur*) and dried. Wheat straw was dried and chopped to a 2.5-5 cm particle size. All substrates were watered to 60% moisture and used to fill 0.5–L glass jars.

Jars with all substrate variants (60±1.5 g of wet substrate in each) were autoclaved for 90 minutes at 121°C and inoculated with 25-day−old mycelium grown on MEA at 26±0.1°C. In each jar, 1/4 part of the mycelial colony was inserted from a 90-mm Petri dish. The jars were incubated at 26±0.1°C. Every day, the degree of substrate mycelial colonization was monitored and recorded visually. To achieve this goal, we measured the height of the substrate, which was covered with mycelium from four mutually perpendicular sides of the container. The average values were calculated, and the (in %) height of the substrate colonized by mycelium was determined.

Statistical analysis

All experiments were independently conducted in three replicates. The results were processed using Statistica 8.0 (StatSoft Inc., USA), x ± y represents mean standard deviation in all cases. Significant differences between values are indicated at $P \leq 0.05$ level.

***Re-situ* method in the natural environment**

To reintroduce *S. laminosa* into natural conditions, we laid several mycological reproduction sites in the territory of the Hutsulshchyna National Nature Park in June 2018. According to the zoning of the park, we placed the mycological reproduction area № 1 in the zone of regulated recreation (the territory of the village of Verbovets) in the temperate deciduous forest dominated by common beech (*Fagus sylvatica* L.), common hornbeam (*Carpinus betulus* L.) and common oak (*Quercus robur*), with some pines (*Pinus sylvestris*) and silver fir (*Abies alba*) (Table 2).

Table 2 GPS data on mycological reproduction sites for *Sparassis laminosa* at the reproduction area № 1

Names of the substrates	Coordinates	Locations for each type of substrate		
		1 spot	2 spot	3 spot
1 WgCsSs	Latitude, N	48°21′03.7″	48°21′04.9″	48°21′05.2″
	Longitude, E	025°08′34.8″	025°08′39.7″	025°08′40.5″
2 WgCsSsWs	Latitude, N	48°21′05.2″	48°21′04.4″	48°21′04.4″
	Longitude, E	025°08′42.2″	025°08′40.5″	025°08′39.8″
3 WgPsSsPns	Latitude, N	48°21′04.9″	48°21′04.7″	48°21′05.1″
	Longitude, E	025°08′36.5″	025°08′37.7″	025°08′39.5″
7 OlWg	Latitude, N	48°21′04.4″	48°21′04.6″	48°21′05.0″
	Longitude, E	025°08′36.1″	025°08′37.3″	025°08′39.6″
8 OlCsWg	Latitude, N	48°21′04.3″	48°21′05.0″	48°21′03.6″
	Longitude, E	025°08′35.7″	025°08′37.0″	025°08′34.9″
9 OlWgWs	Latitude, N	48°21′05.1″	48°21′05.0″	48°21′04.2″
	Longitude, E	025°08′38.2″	025°08′36.6″	025°08′35.7″
10 CsOlWgWs	Latitude, N	48°21′05.3″	48°21′05.4″	48°21′03.9″
	Longitude, E	025°08′38.4″	025°08′36.7″	025°08′35.2″
11 Sunflower	Latitude, N	48°21′05.3″	48°21′04.3″	48°21′03.7″
	Longitude, E	025°08′38.2″	025°08′36.1″	025°08′34.8″
12 SsCs	Latitude, N	48°21′05.1″	48°21′04.2″	48°21′03.5″
	Longitude, E	025°08′38.0″	025°08′36.3″	025°08′34.5
13 Sawdust	Latitude, N	48°21′04.9″	48°21′04.7″	48°21′03.4″
	Longitude, E	025°08′37.8″	025°08′38.5″	025°08′34.2″
14 Bamboo	Latitude, N	48°21′05.0″	48°21′04.9″	48°21′03.1″
	Longitude, E	025°08′38.0″	025°08′39.3″	025°08′34.6″
15 Wheat	Latitude, N	48°21′04.9″	48°21′05.2″	48°21′03.3″
	Longitude, E	025°08′38.1″	025°08′39.4″	025°08′35.0″

In the natural conditions, we introduced the mycelium of *S. laminosa* grown on the substrates № 1 WgCsSs, № 2 WgCsSsWs, № 3 WgPsSsPns, № 7 OlWg, № 8 OlCsWg, № 9 OlWgWs, № 10 CsOlWgWs, № 11 Sunflower, № 12 SsCs, № 13 Sawdust, № 14 Bamboo, № 15 Wheat. To achieve this goal, the substrate with mycelium was placed into the top soil layer of sites located within 0.5-1 m from the trunks of oak (*Q. robur*), onto the roots of trees with trunks at least 40 cm in diameter. The locations of these sites were marked with pins of different colors and/or marks on surrounding objects, GPS data were established by Garmin Etrex 20x. The sites were moistened once with a solution of streptomycin (1 g-/-5 L of water). Later, we moistened the soil of the sites with warm (20°C) water for two weeks. In total, we placed 36 spots for mycological reproduction site № 1 (Table 2), with three locations for each type of substrate used in the experiment.

Mycological reproduction site № 2 was established in the arboretum of the Hutsulshchyna National Nature Park, village of Stari Kuty. This area of 0.37 hectares is home for more than 200 species of trees and shrubs (Anonymous 2010–2017), including seven oak species: common oak *Q. robur*, Strandzha oak *Q. hartwissiana* Stev, sessile oak *Q. petraea* Liebl, scarlet oak *Q. coccinea* Munchh, Austrian oak *Q. austriaca* Willd, pin oak *Q. palustris* L., and red oak *Q. rubra* L.

The mycelium of *S. laminosa* grown on substrates № 1 WgCsSs, № 2 WgCsSsWs, № 3 WgPsSsPns, № 7 OlWg, № 8 OlCsWg, № 9 OlWgWs was introduced in the same way as described for common oak *Q. robur* (three spots for each substrate, 9 trees); mycelium grown on substrates № 10 CsOlWgWs, № 11 Sunflower, № 12 SsCs, № 13 Sawdust, as described for rock oak *Q. petraea* in three spots (two trees); mycelium on the substrates № 14 Bamboo, № 15 Wheat for Gartwis oak *Q. hartwissiana* in three spots (two trees, Table 3). No experiments were conducted with other oak species because of the small diameter of their trunks (less than 40 cm). Moistening and marking was conducted in the same way in all locations. Monitoring of the laying sites was carried out weekly until the formation of a stable snow cover, up to November 2018.

Table 3 GPS data on mycological reproduction sites for *Sparassis laminosa* at the reproduction area № 2

Names of the substrates and specie of tree	**Coordinates**	**Locations for each type of substrate**		
		1 spot	**2 spot**	**3 spot**
1 WgCsSs	Latitude, N	48°17′16.6″	48°17′16.6″	48°17′16.8″
Q. robur	Longitude, E	025°10′15.5″	025°10′15.5″	025°10′12.6″
2 WgCsSsWs	Latitude, N	48°17′17.2″	48°17′17.3″	48°17′17.2″
Q. robur	Longitude, E	025°10′13.0″	025°10′12.6″	025°10′12.4″
3 WgPsSsPns	Latitude, N	48°17′17.3″	48°17′17.3″	48°17′17.1″
Q. robur	Longitude, E	025°10′12.3″	025°10′12.1″	025°10′11.7″
7 OlWg	Latitude, N	48°17′17.0″	48°17′17.4″	48°17′17.4″
Q. robur	Longitude, E	025°10′11.7″	025°10′11.1″	025°10′10.9″
8 OlCsWg	Latitude, N	48°17′17.1″	48°17′17.1″	48°17′17.4″
Q. robur	Longitude, E	025°10′10.4″	025°10′10.3″	025°10′10.0″
9 OlWgWs	Latitude, N	48°17′17.4″	48°17′17.7″	48°17′17.6″
Q. robur	Longitude, E	025°10′09.9″	025°10′09.7″	025°10′09.6″
10 CsOlWgWs	Latitude, N	48°17′16.8″	48°17′16.8″	48°17′16.8″
Q. petraea	Longitude, E	025°10′13.2″	025°10′13.2″	025°10′13.3″
11 Sunflower	Latitude, N	48°17′16.8″	48°17′16.8″	48°17′16.8″
Q. petraea	Longitude, E	025°10′13.2″	025°10′13.2″	025°10′13.2″
12 SsCs	Latitude, N	48°17′16.8″	48°17′16.7″	48°17′16.7″
Q. petraea	Longitude, E	025°10′13.2″	025°10′13.2″	025°10′13.1″
13 Sawdust	Latitude, N	48°17′16.8″	48°17′16.7″	48°17′16.7″
Q. petraea	Longitude, E	025°10′13.2″	025°10′13.3″	025°10′13.3″
14 Bamboo	Latitude, N	48°17′16.6″	48°17′16.6″	48°17′16.6″
Q. hartwissiana	Longitude, E	025°10′15.5″	025°10′15.5″	025°10′15.5″
15 Wheat	Latitude, N	48°17′15.7″	48°17′15.7″	48°17′15.7″
Q. hartwissiana	Longitude, E	025°10′15.2″	025°10′15.2″	025°10′15.2″

Results

Mycelial features of *Sparassis laminosa* 2211

The morphology of *S. laminosa* mycelial colonies on nutrient agar media of different composition. On all the used media, dense, opaque, white wooly colonies formed with cottony aerial mycelium. The edge of the colony was slightly raised above the substrate, odor was absent, and the color of the reversal was the same as the color of the medium (Fig. 1). The minimum colony growth rate – 2.5±0.02 mm/day – was observed on PDA, and the maximum – 3.5±0.03 mm/day – on MEA (Fig. 2).

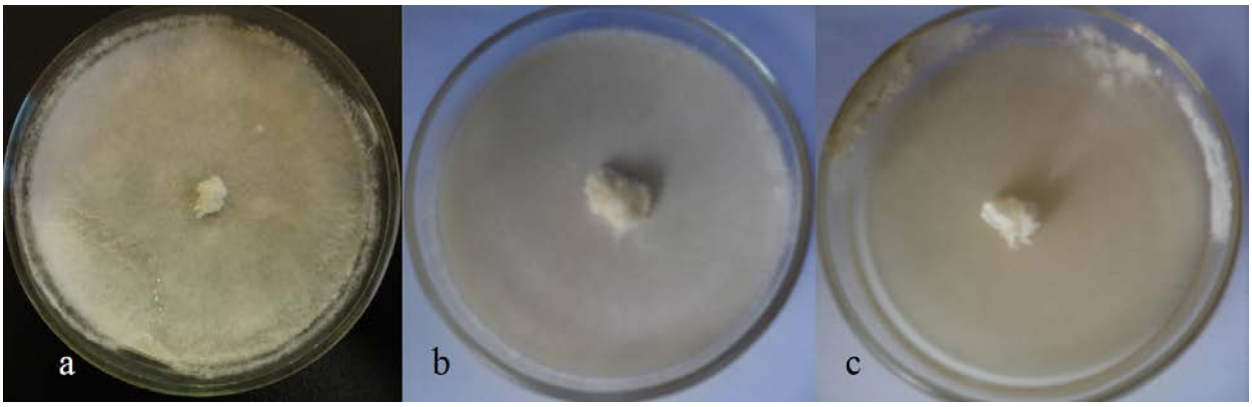

Fig. 1 – Mycelial colonies of *Sparassis laminosa* 2211 on agar PDA–nutrient medium (a), MEA (b) and MAA (c) at 26±0.1°C on day 14 of mycelial growth (three replicates).

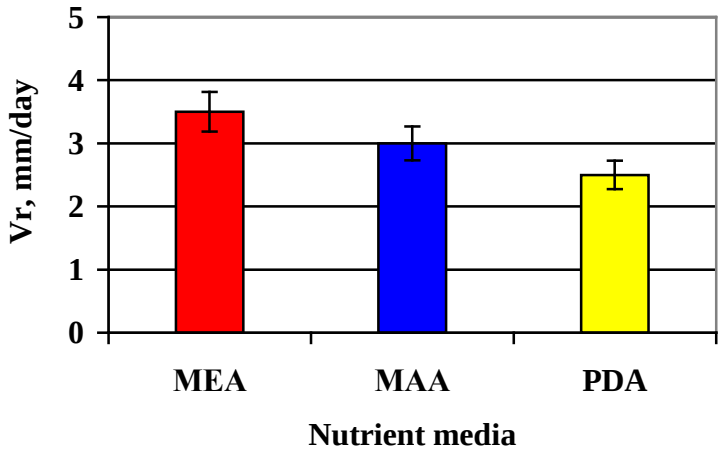

Fig. 2 – Radial rate of growth of *Sparassis laminosa* 2211 colonies on three nutrient agar media at 26±0.1°C, for 14 days.

Adaptation of the mycelia of *S. laminosa* 2211 on different substrates

The second stage of the experiment consisted of obtaining the inoculated mycelium on different substrates. Among the fifteen tested substrates, four substrates including mixture of wheat grain: conifer sawdust: sunflower seed shells (№ 1 WgCsSs, ratios listed components were 40%:40%:20%), mixture of wheat grain: conifer sawdust: sunflower seed shells: wheat straw (№ 2 WgCsSsWs, ratios listed components were 67%:17%:8%:8%), mixture of wheat grain: sunflower seed shells: pumpkin seeds shells: peanut shells (№ 3 WgPsSsPns, ratios listed components were 50%:12.5%:25%:12.5%), and № 15 Wheat (wheat formed 100% of mass), were completely colonized by mycelium as early as days 10-20. The highest degree of colonization of substrates № 11 Sunflower (seed shells formed 100% of mass), № 12 SsCs (conifer sawdust was 9 % of mass, sunflower seed shells were 91% of mass), № 13 100% Sawdust, № 14 100% Bamboo occurred after days 25-30 of the experiment. On day 30 substrates № 11, 12, 13 and 14 were overgrown by mycelium accordingly on 100±5%, 95±5%, 82±5% and 62±5 % (Fig. 3).

On day 35 of the experiment substrates № 7 OlWg (grain was 16.7% of mass, oak forest litter was 83.3% of mass), № 8 OlCsWg (ratios components grain: sawdust: litter were 9%:45.5%:45.5% of mass), № 9 OlWgWs (ratios components grain: straw: litter were 14.3%:14.3%:71.4% of mass), № 10 CsOlWgWs (ratios components grain: sawdust: straw: litter were 16.7%:33.3%:16.7%:33.3%) were colonized minimum on 21±4% (substrate № 8) and maximum

on 52±6% (substrate № 9). Substrates № 4 CsSs, 5 CsSsWs, 6 PsSsPns did not contain wheat grain at all and were not suitable for mycelial growth (Fig. 3).

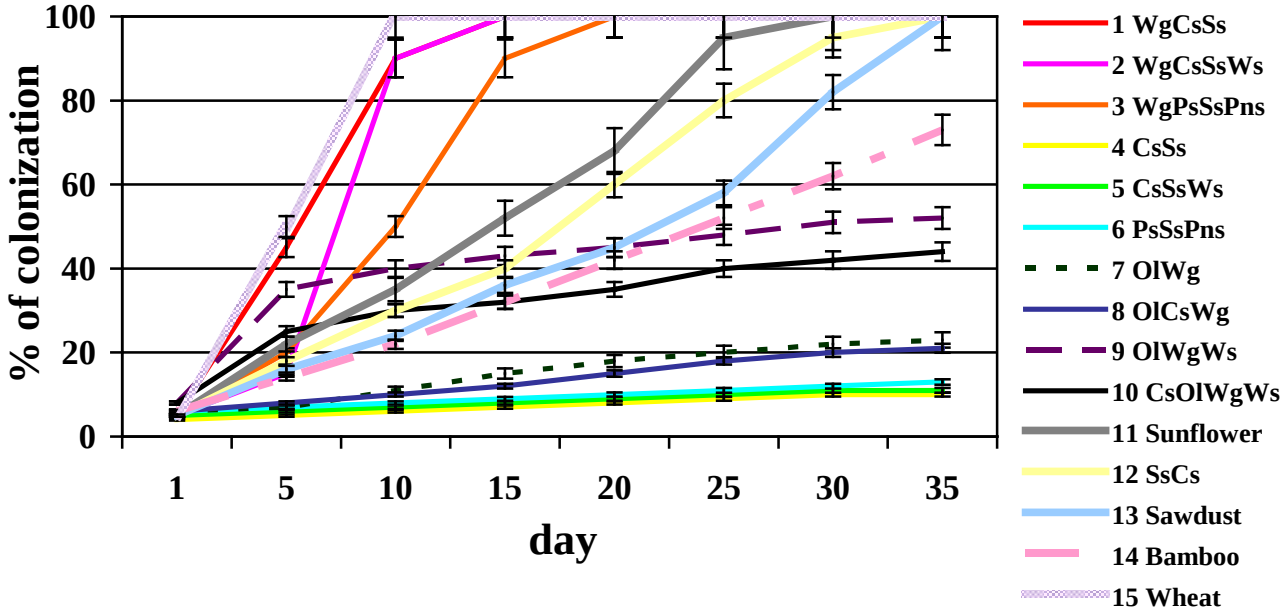

Fig. 3 – Dynamics of substrate colonization by the mycelium of *Sparassis laminosa* 2211

Notes – Differences are significant between substrates № 1, 2, 3, 15 and other substrates on days 15 and 20 of experiment, P≤0.05; & – Differences are significant between substrates № 4, 5, 6 and other substrates on days 20, 25, 30, 35 of experiment, P≤0.05.

The significantly lower grain content in substrate combinations № 7 (16.7%), № 8 (9%), № 9 (14.3%) and № 10 (16.7%) compared with substrates № 1 (40%), № 2 (67%) and № 3 (50%) again confirmed that wheat grain was necessary for successful mycelial growth. The presence of other components used in the experimental substrates appeared to be less important. The mycelium successfully colonized substrates with various additives (conifer sawdust, forest litter, straw, peanuts shells) if the grain was part of the mixture

No growth was observed on the substrates № 4 CsSs, 5 CsSsWs, 6 PsSsPns (amounts of grain were 0%). However, the inclusion of large amounts of grain to the components of substrates № 4–6 (resulting in substrates № 1 WgCsSs, № 2 WgCsSsWs, № 3 WgPsSsPns) considerably and statistically significant increased the intensity and percentage of their colonization (Fig. 3, P≤0.05). The slow mycelial growth on substrates № 4 CsSs, 5 CsSsWs, 6 PsSsPns was unexpected because the composition of these substrates included the shells of sunflower. Substrates № 11 Sunflower (shells of sunflower seeds formed 100 % of mass) and № 12 SsCs (ratios coniferous sawdust: sunflower seed shells were accordingly 9%:91%) were completely colonized at days 30-35 of the experiment (Fig. 3). We considered the important physical criterion for using a substrate to be the degree of shredding and, probably, the density of the mixture of sawdust and shells.

Thus, the *S. laminosa* culture could grow successfully on substrates with wheat grain, including atypical components for Ukrainian flora – shells of peanut. Coniferous sawdust was not an obstacle for mycelial growth of *S. laminosa*, on which fruit bodies more often grew near oaks than coniferous trees. This feature might be strain specific, similar to the reported ability of strain 2211 of *S. laminosa* to colonize pine sawdust (Tsvyd et al. 2016). Interestingly, the mycelium of *S. laminosa* 2211 was also capable of growing on bamboo sticks (substrate №14 Bamboo, 73±5 % colonized).

On substrates № 1 WgCsSs, № 2 WgCsSsWs, the vegetative mycelium formed white, dense colonies with even edges pressed against the surface (Fig. 4 a, b, c, d). On the surface of the colonies we observed some aerial hyphae, which showed superior expression on pumpkin seed shells (substrate № 3 WgPsSsPns, Fig. 4e). On substrates № 7 OlWg, № 8 OlCsWg, № 9 OlWgWs, № 10 CsOlWgWs with incomplete mycelial colonization, the color and texture were the same as on other substrates, with some clearly visible substrate particles, which were not covered with mycelium (Fig. 4f).

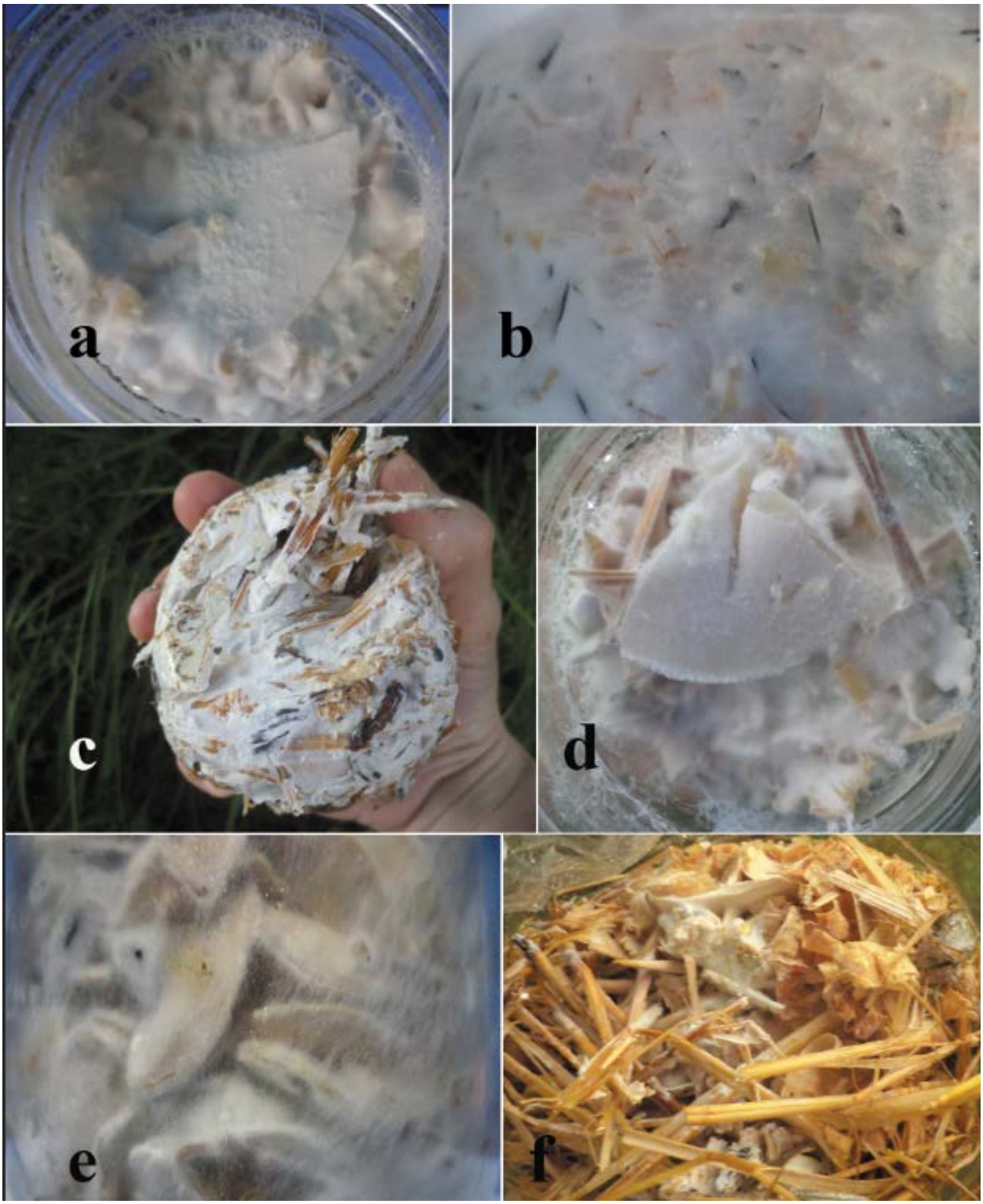

Fig. 4 – Mycelium of *Sparassis laminosa* 2211 on the combined substrates: a, b № 1 WgCsSs. c, d № 2 WgCsSsWs. e – № 3 WgPsSsPns. f №10 CsOlWgWs on day 50 at 26±0.1ºC.

Fruiting of *S. laminosa* 2211 under laboratory conditions was observed only on substrates № 11 Sunflower, № 12 SsCs, № 13 Sawdust (Fig. 5).

The mature fruit bodies displayed a white or cream color. Several fruit bodies (2-5) formed on a certain amount of the substrate with a typical morphology, but they had a smaller size (from one to several cm). The fastest growing (on day 33 of the experiment) fruit bodies formed on the substrate from sunflower shells (№ 11 Sunflower), whereas on the substrates № 12 SsCs and № 13 Sawdust, fruit body formations required 67 and 91 days, respectively.

Fig. 5 – Successful fructification of *Sparassis laminosa* 2211 occurred on the following: a Day 33 on substrate № 11 Sunflower. b Day 67 on substrate № 12 SsCs. c, d Day 91 on substrate № 13 Sawdust at 26±0.1°C, 60 % humidity.

***Re–situ* method in nature**

The third stage of our studies were conducted under natural conditions. The substrates colonized with mycelium of *S. laminosa* (except № 4 CsSs, № 5 CsSsWs, № 6 PsSsPns) were transferred to a natural environment after the occurrence of rain. For two weeks, the spots of their introduction were moistened daily with water from a well warmed to 20°C and monitored. We observed fruiting of *S. laminosa* only in the case of substrate № 2 WgCsSsWs, despite the absence of fructification on this substrate under laboratory conditions. The fruiting occurred in four months after introduction of the substrate to single site (GPS data are N 48°21′05.2″, E 025°08′42.2″) at the reproduction area № 1 near the village Verbovets in September 2018.

The obtained fruit bodies of *S. laminosa* were irregular, first resupinant and later semi-globose, and initially white and later creamy colored. Their maximum diameter was 20 cm, with a height of 1-2 cm. The lobes had a width 0.5-1 cm, elongated from 1-1.5 cm, and they were flattened and smooth. The edges of the lobes were curvy (Fig. 6). Thus, the fruit bodies were typical of this species, but significantly smaller.

Analysis of the reproduction results for the fungus in nature highlights important components, the combination of which led to a positive result – actual fruiting. These components include abiotic factors, such as the composition of the substrate, weather, or moisture content of the soil, as well as biotic factors, such as the: percent colonization of the substrate compared with its whole volume, and competitive fungi. One challenge that we encountered during the introduction of *S. laminosa* using the *re-situ* method – was contamination with subsequent fructification of a widespread basidiomycete, *Schizophyllum commune* Fr. This basidiomycete is found only on rotting wood, not on soil. We assume that the substrates we used had been infected by this fungus, which inhibited or replaced *S. laminosa*. Since fruit bodies of *S. commune* were observed only in places where the substrates № 7 OlWg, № 8 OlCsWg, № 9 OlWgWs, № 10 CsOlWgWs were used (Fig. 7), these substrates might not be suitable for further use for the reintroduction of *S. laminosa* in nature. The most plausible reason for the appearance of *S. commune* fruit bodies is incomplete substrate colonization by *S. laminosa*.

Fig. 6 – Fruit body of *Sparassis laminosa*, obtained in the natural environment.

Fig. 7 – Fruit bodies of *Schizophyllum commune* on the ground, at the site of introduction of *S. laminose.*

Discussion

Work with pure cultures of macromycetes requires continuous control of their purity using the established macromorphological features of a certain species (Nobles 1971). The most

important taxonomic control feature for the identification of basidial macromycetes in pure culture is the stage of the teleomorphs, which we observed on MEA at day 15 of cultivation MEA was the most suitable medium for growing *S. laminosa* 2211 mycelium, as the growth rates in this culture were the highest compared with the other media used. The unpretentiousness the *S. laminosa* culture to media was shown also in the work Krivko & Bondarenko 2004. The *S. laminosa* culture grew on potato-glucose agar, potato-carrot-glucose agar and pumpkin-glucose agar, completely covering the space of Petri dishes on day 5 of the experiment. Using of monosaccharides (glucose, fructose, maltose) in this experiment provided more intensive growth the *S. laminosa* culture compared to disaccharides (lactose). Since 1975 *Sparassis laminosa* (and *S. crispa)* are described in culture following the method used by Nobles. *Sparassidaceae* are a very active wood destroying fungi in blocks of Douglas fir (sapwood and heartwood) in laboratory tests. The cauliflower-like fructifications are easily produced on malt-agar and could be produced on a large scale (Delatour 1975).

Surprisingly the addition of fir sawdust to the nutrient medium did not improve the growth characteristics of the culture but, in contrast, inhibited colony growth. Considering 2.5-3.5 mm/day of radial growth rate on all selected media the *Sparassis laminosa* 2211 culture was a slow-growing culture (2-4 mm/day). *S. laminosa* 2211 culture was simultaneously adaptive to the cultivation conditions, because all tested media supported its growth. In addition, the colonies maintained their vitality over four months after complete colonization of the medium surface. In this case, typical signs of aging (drops of exudate and/or brownish pigmentation) that occur in the long-term storage of species such as *Fomes officinalis* (Vill.) Bres. 5004 (Mykchaylova et al. 2017), *Coriolus zonatus* (Nees) Quél. 5300, *C. versicolor* (L.) Quél. 5129 (Klechak et al. 2008) were not observed.

Cultivation of *Sparassis laminosa* is not well studied but promising for the purpose of cultivating it as an edible species. The rare occurrence of this species in nature makes it relevant to study reintroduction of species as rare and endangered species of basidiomycetes. Cauliflower mushroom (*S. crispa*) is being cultivated as a functional mushroom since the mushroom contains larger amount of β-glucan than other edible or medicinal mushrooms (Park et al. 2011). In the study were assayed the mycelial growth and the productivity of the mushroom cultivated on the sawdust-based medium made of larch (*Larix kaempferi*). The best mushroom productivity was better at sawdust-based media density 0.76 g/cm^3 than any other densities (0.68-0.80 g/cm^3) with excluding the particles less than 1 mm.

The degree of shredding (and the density of the substrate) was the important physical criterion for using the substrates for cultivating *Sparassis laminosa* 2211. Dependence successfully using of substrates to their density we too observed for *Clathrus archeri* (Berk.) Dring, where the positive effect on the mycelium growth had been caused by the improved aeration of the substrate and lower density of it (Pasailiuk et al. 2018). The mycelium of *S. laminosa* showed successful growth on substrates with the higher degree of shredding (№ 1 WgCsSs – wheat grain: conifer sawdust: sunflower seed shells, № 2 WgCsSsWs – wheat grain: conifer sawdust: sunflower seed shells, № 3 WgPsSsPns – wheat grain: pumpkin seed shells: sunflower seed shells: peanut shells, № 15 Wheat) compared with the lower degree of grinding (substrates № 7 OlWg – oak forest litter: wheat grain, № 8 OlCsWg – oak forest litter: conifer sawdust: wheat grain, № 9 OlWgWs – oak forest litter: wheat grain: wheat straw, № 11 Sunflower). Therefore, the best mycelium growth of *S. laminosa* 2211 was at the substrates higher degree of shredding.

It is established too that wheat grain can be considered an important component for the successfully cultivation of *S. laminosa* 2211 mycelium. It is not surprising because wheat grain contains useful nutrient components and it is maybe important for successfully growth the mycelium in laboratory condition.

That's why among the tested solid substrates, the fastest mycelial growth was observed on 100% wheat grain (substrate № 15 Wheat) – the fungus completely colonized the grain at day 10 after inoculation. Rapid growth rates of the mycelium were observed on the grain–containing the combined substrates № 1 WgCsSs (grain is 40% of mass), № 2 WgCsSsWs (grain is 67% of mass) and № 3 WgPsSsPns (grain is 50% of mass) with complete colonization from days 15-20.

Conversely, the same substrate without the grain component (№ 4 CsSs, № 5 CsSsWs, № 6 PsSsPns) showed colonization of less than 13±2%. Despite the rapid growth on substrates № 1 WgCsSs, № 2 WgCsSsWs and № 3 WgPsSsPns, even the following prolonged cultivation (150 days) was not sufficient for the formation of fruit bodies or their primordia, perhaps due to an insufficient total amount of substrate per one vessel (60 g). The exudate and yellowish mycelium (but not fruiting) were observed two months after complete colonization of the substrates.

Fruiting of *Sparassis laminosa* we observed on sunflower seed shells (substrate № 11), sawdust (substrate № 13) and their mix (substrate № 12). So, mass (60 g) listed substrates is sufficient for successful fructification.

In the environment, we observed *S. laminosa* fruiting only on substrate № 2 WgCsSsWs – wheat grain: conifer sawdust: sunflower seed shells: wheat straw. No fruiting in nature was observed on substrates such as sunflower seed shells (№ 11 Sunflower), coniferous sawdust (№ 13-Sawdust) and № 12 SsCs – a combination of sunflower seed shells – coniferous sawdust, despite fructification on these substrates under laboratory conditions. It is possible that the high nutritional value of these substrates triggered some unknown biotic factors, which prevented fructification of the mycelium. Successful fructification on substrate № 2 WgCsSsWs in nature might have resulted from the substrate density in addition to its complete and rapid colonization by the mycelium of *S. laminosa*. Unlike the others, substrates № 1 WgCsSs and № 2 WgCsSsWs preserved the shape of their vessels after releasing them the high density of substrate № 2 WgCsSsWs might have preserved the mycelium from possible damage during transportation and handling.

The *re-situ* is a method that provides introducing and support of vital functions of mushroom in nature with the forming of their basidioma. However, no studies have examined the possible reproduction of *S. laminosa* under natural conditions, but we practiced to grow *Clathrus archeri* in nature (Pasailiuk et al. 2018). New method of preserving the rare species of fungi in nature was used for *Hericium coralloides* (Scop.) Pers. – oak dowels colonised with *H. coralloides* (obtained from fruit body tissue isolation) were inoculated into 15 standing living beech trees and 3 ash trees (Crockatt 2008).

Thus, we showed the possibility of *S. laminosa* reproduction in the territory of Hutsulshchyna national Natural Park.

Conclusion

Peculiarities of *Sparassis laminosa* 2211 culture growth on nutrient agar media and compositions of various plant substrates were studied. MEA was the best agar medium to support mycelial growth. However, PDA and MAA, supplemented by fir sawdust were also suitable for culturing *S. laminosa* under laboratory conditions.

Grain and grain-containing substrates were optimal for spawn production. The mycelium completely colonized substrate № 15 Wheat (content of wheat is 100 % of mass) over day 10 of the experiment. Grain-containing substrates № 1 WgCsSs (wheat grain 40%: coniferous sawdust 40%: sunflower seed shells 20%), № 2 WgCsSsWs (wheat grain 67%: coniferous sawdust 17%: sunflower seed shells 8%: wheat straw 8%) and № 3 WgPsSsPns (wheat grain 50%: pumpkin seed shells 12.5%: sunflower seed shells 25%: peanut shells 12.5%) were completely overgrown by mycelium accordingly over days 15, 15 and 20 of the experiment. We found that only the substrates that were completely colonized by *S. laminosa* mycelium were suitable for application in nature. Partially colonized substrates were contaminated and produced fruit bodies of *Schizophyllum commune* at the site of introduction into nature. Fruiting of *S. laminosa* was observed only in one location, after spawning in the soil under *Q. robur* within a sunny clearing in a temperate deciduous forest, near the village Verbovets, which occurred at four months after introduction of the mycelium on combined substrate № 2 WgCsSsWs (wheat grain 67%: coniferous sawdust 17%: sunflower seed shells 8%: wheat straw 8%). The morphology of the formed fruit bodies was typical of the species, but with a considerably smaller size. The experimental sites continue to be monitored, allowing a general determination of the periodicity of fruiting to identify the effectiveness of the substrates for the re-introduction of *S. laminos*a 2211 in nature.

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