

INFLUENCE OF CULTURE SUBSTRATE pH ON CORDYCEPS MILITARIS MUSHROOM CORDYCEPIN CONTENT, GROWN ON DIFFERENT SOLID SUBSTRATES

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Abstract

The Tibetan mushroom (*Cordyceps militaris*) has been known and used in traditional Chinese medicine for its therapeutic properties for thousands of years. This mushroom is rich in nutrients and biologically active components, such as: cordicepic acid, glutamic acid, mannitol, proteins, amino acids, polysaccharides, magnesium and calcium. These substances make Tibetan Mushroom an extraordinary means of restoring health and increasing the vitality of the body. In the study, we used a solid culture substrate with 3 degrees (based on brown rice, wheat and barley) with a pH of 7 degrees (4.5-7.5). It was found that the pH of the culture substrate directly influences the production of cordycepin, so at a pH value of 5 the cordycepin content begins to increase, reaching its highest level at a pH value of 5.5 (362 mg/L), then at pH values above 6, the cordycepin concentration begins to decrease.

Keywords: cordycepin, *Cordyceps militaris*, mushroom, substrate pH

1. INTRODUCTION

The Tibetan mushroom (*Cordyceps militaris*) belongs to the *Ascomycetes* class, and is known, along with many other mushroom species, to possess numerous medicinal properties (Rozsa et al., 2016a, b, c, 2017a, b). This species is rich in nutrients and biologically active components, such as: cordicepic acid, glutamic acid, mannitol, proteins, amino acids, polysaccharides, magnesium and calcium (Kalyoncu et al., 2010; Mohd et al., 2017).

The *Cordyceps* mushroom is popularly referred to as the "caterpillar mushroom". In the warm season, it has a development cycle similar to that of mushrooms, and in the cold season it becomes a host for the larvae of certain species of insects (Patel and Ingalhali, 2013). In nature, to survive, the fungus kills and mummifies the insect, feeding on its body.

Cordyceps grows in Tibet and Nepal at altitudes between 3,000 and 5,000 meters. This mushroom was discovered about 1,500 years ago by Himalayan shepherds and soon became an herbal remedy with powerful energizing, anti-aging, dementia-preventing and toning effects (Wang and Shiao, 2000; Rathore et al., 2019).

Its rarity and miraculous properties have made it one of the most expensive cures. In Ancient China, only members of the imperial court and the high aristocracy had access to cordyceps (Yang et al., 1994; Xiao and Zhong, 2007). Currently, this mushroom is cultivated under special conditions, the annual production increasing from year to year, the host insects being replaced by various

projective additions in the substrate, and its extraordinary properties can now benefit as many people as possible (Singpoonga et al., 2020; Rozsa et al., 2022)




Cordycepin and adenosine are the main ingredients of cordyceps, the quality of a culture being determined by the concentration of these active substances (Zhu et al., 1998). Cordycepin and adenosine are involved in a large number of cellular biological processes, such as inter- and intra-cellular energy transfer (Rozsa et al., 2017c, d, e, f).

2. MATERIALS AND METHODS

A pure mycelium culture containing the TL7001 strain obtained from SC Ciupercaria SRL mycelium research laboratories was used in the experiment.

Cereal grains were used as culture substrate, this experience factor presenting 3 gradations (1 – brown rice, 2 – wheat and 3 – barley). For pH, 7 gradations were used, with pH values starting from 4.5 and up to 7.5 in 0.5-point increments (Rozsa et al., 2016d, e). From the combination of experimental factors, as can be seen in Table 1, 21 experimental variants resulted.

Table 1. Experimental factors

Experimental variant	Substrate	pH – value
V1	S1 - Brown rice 	P1 - 4,5
V2		P2 - 5
V3		P3 - 5,5
V4		P4 - 6
V5		P5 - 6,5
V6		P6 - 7
V7		P7- 7,5
V8	S2 – Wheat 	P1 - 4,5
V9		P2 – 5
V10		P3 - 5,5
V11		P4 – 6
V12		P5 - 6,5
V13		P6 – 7
V14		P7- 7,5
V15	S3 – Barley 	P1 - 4,5
V16		P2 – 5
V17		P3 - 5,5
V18		P4 – 6
V19		P5 - 6,5
V20		P6 – 7
V21		P7- 7,5

The optimal initial grain moisture content for inoculum production is between 49-54% (Rozsa et al., 2016f).

In all grain recipes, chalk (CaCO₃) and gypsum (CaSO₄) were added at a rate of 1-3 parts by weight per 100 parts of grain (dry weight) (Rozsa et al., 2019). The ratio of chalk to gypsum is 1:4. Peptone was used as a protein additive, and the pH of the substrates was adjusted with citric acid (C₆H₈O₇) 0.1N or with sodium hydroxide (NaOH) 0.1N, depending on the experimental variant (Rozsa et al., 2016g).

The grains were moistened to 48-52% humidity, then placed in jars with a capacity of 1 liter (Figure 1), sterilized in an autoclave for 1 hour at 121 °C. After cooling, they were inoculated with 1 cm² of mycelium from pure culture on agar medium (Rozsa et al., 2016h).



Figure 1. Preparation of the culture substrate in jars with a capacity of 1 liter

Incubation and growth of the mycelium lasted 21 days at 23 °C, with the jars being shaken every third day. Cordycepin determinations were made on the 22nd day after inoculation (Figure 2).



Figure 2. Appearance of culture medium before cordycepin determination

The determination of cordycepin was carried out after its extraction from the mycelial biomass with the help of methanol, in a ratio of 1:5, the samples being sonicated for 15 min. Then after centrifugation for 15 min, the sample was filtered through a 0.2 µm filter, then the sample was placed in a microcentrifuge tube. HPLC analysis was performed using a C18 column, the retention time of cordycepin was measured at λ max 260 nm using a UV detector.

The experiment was carried out in three repetitions, and the data obtained were statistically processed using Duncan's test.

3. RESULTS AND DISCUSSIONS

Following the unilateral influence of the substrate on the content of cordycepin in the *Cordyceps militaris* mushroom, taking as a control the substrate with wheat grains, which in the current practice of obtaining mycelial biomass is used on a large scale (Rozsa et al, 2016a, b, c), it can be observed that from a statistical point of view, there are no differences compared to the S1 substrate, instead the S3 substrate registered significantly negative differences compared to the control variant, the data being presented in Table 2.

Table 2. Unilateral influence of the substrate on the content of cordycepin in the *Cordyceps militaris* mushroom

Substrate composition	Cordycepin production mg/L		Difference ±D	Signification of difference
	Obtained values	%		
S2	282.48 a	100.0	0.00	Control
S1	292.05 a	103.4	9.57	-
S3	263.57 b	93.3	-18.90	00

SD (Duncan test) 10.41-10.62

LSD (p 5%) 10.41
LSD (p 1%) 17.22
LSD (p 0.1%) 32.24

The results obtained by us are comparable to those presented by Mohd et al. 2017.

In order to follow the unilateral influence of pH on the cordycepin content in the *Cordyceps militaris* mushroom substrate, the average of the experience (Rozsa et al., 2016e) was considered as a control, the data obtained being presented in Table 3.

Following the data presented in Table 3, it can be seen that variant P3 (pH=5.5) recorded the highest value, 354.22 mg/L, with a very significantly positive difference of 74.86 mg/L compared to the control, being followed by variant P4 with a distinctly significant positive difference of 31.08 mg/L compared to the experience control and variant P2 with a positive difference of 19.63 mg/L compared to the experience control. In the experimental variants P1 and P5, no statistically assured values were recorded, and the experimental variants P6 and P7 recorded very significantly negative values compared to the average of the experience considered as a control.

Table 3. Unilateral influence of pH on the cordycepin content in the *Cordyceps militaris* mushroom substrate

Substrate pH value	Cordycepine production mg/L		Difference ±D	Signification of difference
	Obtained values	%		
P0	279.37	100.0	0.00	Control
P1	271.33 d	97.1	-8.03	-
P2	299.00 bc	107.0	19.63	*
P3	354.22 a	126.8	74.86	***
P4	310.44 b	111.1	31.08	**
P5	283.89 cd	101.6	4.52	-
P6	243.33 e	87.1	-36.03	000
P7	193.33 f	69.20	-89.03	000

SD (Duncan test) 19.06-21.75

LSD (p 5%) 19.03
LSD (p 1%) 25.49
LSD (p 0.1%) 33.62

The obtained results are supported statistically and if we look at them from the point of view of the combination of factors P (substrate pH) and S (substrate composition), as can be seen in Table 4.

Table 4. Combination of factors P (substrate pH) and S (substrate composition)

Experimental factors	Cordycepin production mg/L		Difference ±D	Signification of difference
	Obtained values	%		
P0 S1	292.05 -	100.0	0.00	Control
P1 S1	270.00 def	92.5	-22.05	-
P2 S1	293.00 cde	100.3	19.63095	-
P3 S1	362.00 a	124.0	69.95	***
P4 S1	335.33 b	114.8	43.29	*
P5 S1	311.67 bc	106.7	19.62	-
P6 S1	287.67 de	98.5	-4.38	-
P7 S1	184.67 h	63.2	-107.38	000
P0 S2	282.48 -	100.0	0.00	Control
P1 S2	268.00 def	94.9	-14.48	-
P2 S2	304.00 cd	107.6	21.52	-
P3 S2	357.67 a	126.6	75.19	***
P4 S2	299.00 cd	105.8	16.52	-
P5 S2	268.00 def	94.9	-14.48	-
P6 S2	249.00 efg	88.1	-33.48	0
P7 S2	231.67 fgh	82.0	-50.81	00
P0 S3	263.57 -	100.0	0.00	Control
P1 S3	276.00 de	104.7	12.43	-
P2 S3	300.00 cd	113.8	36.43	*
P3 S3	343.00 a	130.1	79.43	***
P4 S3	297.00 cd	112.7	33.43	*
P5 S3	272.00 de	103.2	8.43	-
P6 S3	193.33 gh	73.4	-70.24	000
P7 S3	163.67 h	62.1	-99.90	000

SD (Duncan test) 19.06-21.75

LSD (p 5%) 19.03
LSD (p 1%) 25.49
LSD (p 0.1%) 33.62

Following the data presented in Table 4, it can be observed that the experimental variants P3 S3, P3 S2 and P3 S1, recorded the highest values of cordycepin (343.00-362.00 mg/L). Thus, the importance of the pH value of the culture substrate being highlighted, a fact also confirmed by other authors (Rozsa et al., 2017a, b).

The cordycepin values recorded by us during these experiments are a little lower than those obtained by Singpoonga et al., 2020 and relatively similar to those presented by Lin et al., 2020).

4. CONCLUSIONS

Based on the experimental results obtained regarding the influence of the pH of the culture substrate on the cordycepin content in the *Cordyceps militaris* mushroom, cultivated on different solid substrates, the following can be concluded:

- Regardless of the substrate used, the highest value, 354.22 mg/L, of cordycepin was recorded at the pH value of 5.5.

- In the case of the *Cordyceps* mushroom, the brown rice substrate stimulates a higher production of cordycepin.

5. REFERENCES

- Kalyoncu, F., Oskay, M., & Kayalar, H. (2010). Antioxidant activity of the mycelium of 21 wild mushroom species. *Mycology*, 1(3), 195-199.
- Lin, J.Y., Tsai, H.L., Sang, W.C. (2020) Implementation and Performance Evaluation of Integrated Wireless MultiSensor Module for Aseptic Incubator of *Cordyceps militaris*. *Sensors* 20:15, pages 4272.
- Mohd, A., Syed, A.A., Saif, K., Eyad, A., Amir, M.A. (2017) Effect of pH, temperature and incubation time on cordycepin production from *Cordyceps militaris* using solid-state fermentation on various substrates, *CyTA - Journal of Food*, 15:4, 617-621, DOI: 10.1080/19476337.2017.1325406
- Patel, K. J., & Ingahlalli, R. S. (2013), *Cordyceps militaris* (L.: Fr.) Link-An Important Medicinal Mushroom. *Journal of Pharmacognosy and Phytochemistry*, 2(1).
- Rathore, H., Prasad, S., Kapri, M., Tiwari, A., & Sharma, S. (2019), Medicinal importance of mushroom mycelium: Mechanisms and applications. *Journal of functional foods*, 56, 182-193.
- Rózsa S., (2017a), Correlation between soluble dry matter and β -1,3 d-glucan content in *Agaricus blazei* Murrill mushrooms, *Lucrări Științifice – Seria Horticultură, USAMV Iași, Volume 60 (2)*, 131-138.
- Rózsa S., Dănuț M.N., Gocan Tincuta-Marta, David Stela, Butuza-Bumb Felicia-Suzana, (2016a), Dynamic of the *Agaricus blazei* Murrill mushroom mycelium growth, *Journal of Horticulture, Forestry and Biotechnology, Volume 20(1)*, 120-122.
- Rózsa S., Dănuț M.N., Gocan Tincuta-Marta, David Stela, Butuza-Bumb Felicia-Suzana, (2016b), Research on the biology of the *Agaricus blazei* Murrill mushroom mycelium, *Journal of Horticulture, Forestry and Biotechnology, Volume 20(1)*, 123-126.
- Rózsa S., Dănuț M.N., Gocan Tincuța-Marta, David Stela, Butuza-Bumb Susana-Felicia, (2016c), Research on the influence of temperature on the growth of *Agaricus blazei* Murrill mushroom mycelium, *Agriculture – Science and Practice Journal, Vol 97-98, No 1-2*, 53-57.
- Rózsa S., Dănuț M.N., Gocan Tincuța-Marta, David Stela, Butuza-Bumb Susana-Felicia, (2016d), Influence of pasteurization on different composts used for *Agaricus* ssp. mushroom cultivation on changing the ammonia concentration and the pH level, *Agriculture – Science and Practice Journal, Vol 99-100, No 3-4*, 34-39.
- Rózsa S., Dănuț M.N., Lazăr V., Gocan Tincuța-Marta, Andreica Ileana, (2016e), The influence of pH and the source of nitrogen on the mycelial growth of the *Pleurotus ostreatus* mushrooms, *Scientific works - Agronomy series, USAMV Iași, vol. 59(2)*, 243-246.
- Rózsa S., Dănuț M.N., Lazăr V., Gocan Tincuța-Marta, Butuza-Bumb Susana-Felicia, (2016f), The influence of culture technology on production and chemical content in *Agaricus blazei* Murrill mushrooms, *Scientific works - Agronomy series, USAMV Iași, vol. 59(2)*, 237-242.
- Rózsa S., Dănuț M.N., Poșta G., Gocan Tincuța-Marta, Andreica Ileana, Bogdan Ileana, Rózsa Melinda, Lazăr V., (2019), Influence of the culture substrate on the *Agaricus blazei* Murrill mushrooms vitamins content, *Plants*, 8, 316, <https://doi.org/10.3390/plants8090316>
- Rózsa S., Dănuț M.N., Sima Rodica, Gocan Tincuța-Marta, Andreica Ileana, (2016g), Research on the influence of hybrid, culture substrate and method of disinfection on oyster mushrooms - *Pleurotus* Spp., *Scientific works - Agronomy series, USAMV Iași, vol. 59(1)*, 35-38.
- Rózsa S., Dănuț M.N., Sima Rodica, Gocan Tincuța-Marta, Butuza-Bumb Susana-Felicia, (2016h), Research on the transfer material to obtain mycelium on granular support at the *Agaricus blazei* Murrill mushrooms, *Scientific works - Agronomy series, USAMV Iași, vol. 59(2)*, 231-236.
- Rózsa S., Lazăr V., Gocan Tincuța-Marta, Rózsa Melinda, Poșta G., (2017b), Interaction between growing substrate nitrogen content and *Agaricus blazei* Murrill mushrooms protein content, *Lucrări Științifice – Seria Horticultură, USAMV Iași, Volume 60 (2)*, 123-130.
- Rózsa, S., Dănuț M.N., Gocan, T. M., Sima, R., Lazăr, V., Rózsa, M., (2017c), Influence of temperature and relative humidity on the studded *Agaricus blazei* Murrill mushroom compost, *Current Trends in Natural Sciences*, 6(12), 111-118.
- Rózsa, S., Dănuț M.N., Gocan, T.M., Sima, R., Andreica, I., Rózsa, M., (2017d). *Agaricus blazei* Murrill mushroom compost study anaerobic and aerobic phases. *Current Trends in Natural Sciences*, 6(12), 75-82.

- Rózsa, S., Dănuț M.N., Gocan, T.M., Sima, R., Andreica, I., Rózsa, M., (2017e), Mycelial biomass production of the Sun mushroom (*Agaricus blazei* Murrill), *Current Trends in Natural Sciences*, 6(12), 126-130,
- Rózsa, S., Gocan, T.-M., Lazăr, V., Andreica, I., Rózsa, M., Măniutiu, D.-N., & Sima, R., (2017f), The Effect of Processing on Chemical Constituents of *Agaricus* spp. Mushrooms. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca*, 45(2), 507-516. <https://doi.org/10.15835/nbha45210764>.
- Rózsa, S., Andreica, I., Poșta, G., Gocan, T.M., (2022). Sustainability of *Agaricus blazei* Murrill Mushrooms in Classical and Semi-Mechanized Growing System, through Economic Efficiency, Using Different Culture Substrates. *Sustainability* 14, (10), 6166. <https://doi.org/10.3390/su14106166>
- Singpoonga, N., Sang-on, B., Chairprasart, P., (2020), Effects of culture periods on fruiting body formation and bioactive compounds production of *Cordyceps militaris*. *Acta Horticulturae*:1287, pages 345-352.
- Wang, S.Y. and Shiao M.S., (2000), Pharmacological Functions of Chinese Medicinal Fungus *Cordyceps sinensis* and Related Species: *Journal of Food and Drug Analysis* Vol. 8 No. 4, 248-257.
- Xiao, J.H., Zhong, J.J., (2007), Secondary metabolites from *Cordyceps* species and their antitumor activity studies. *Recent Pat Biotechnol.* 1(2), 123-37.
- Yang, Y.Z., Wang, L.S., Deng, H.Y., (1994), Short-term observation of treating chronic hepatitis B and post-hepatitis cirrhosis with XinGanBao. *Research of Chinese Materia Medica.* 1, 19-20.
- Zhu, J.S., Halpern, G.M. and Jones K., (1998), The scientific rediscovery of an ancient Chinese herbal medicine: *Cordyceps sinensis*: part I and II. *J. Alternative & Complementary Med.* 4, 289-303, 429-457.