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The Effect of Low Temperature Applied During Carpophore Formation on The Development and Nutritional Content of Oyster Mushrooms (*Pleurotus ostreatus* (Jacq. ex Fr.) P. Kumm (1871) and P. citrinopileatus Singer)

Turgay KABAY^{1*}, Aynur SADAK TURHAN ², Suat SENSOY ¹, Ruhan lknur GAZIOGLU SENSOY ¹ ¹ Van Yuzuncu Yil University, Faculty of Agriculture, Department of Horticulture, Van ² Dicle University, Diyarbakir Technical Sciences Vocational School, Diyarbakır *Corresponding author: <u>tkabay@yyu.edu.tr</u>

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Abstract

Consumption of oyster mushrooms, which is a rich food source, has increased in every region of the world. Especially in recent years, there has been an increase in the production and consumption of foods with high nutritional value such as oyster mushrooms. However, the importance of climatic values is very important in mushroom production. In the present study, the growth and nutrient content of two oyster mushrooms [Pleurotus ostreatus (Jacq. ex Fr.) P. Kumm (1871) (gray oyster mushroom), P. citrinopileatus Singer (yellow oyster mushroom)] were investigated under two different temperatures (22 °C and 10 °C after carpophore formation) to see how mushroom would be affected under low temperature conditions. The study, which was established with 2-liter ready-made mushroom production kits, was designed according to the completely randomized experimental plot design with three replications each consisting four kits. Carpophore area and thickness, potassium (K), calcium (Ca) and magnesium (Mg) contents, and the changes in catalase (CAT) and Ascorbate peroxidase (APX) enzyme activities were investigated in both ovster mushroom species under two temperatures. There were various effects of low temperature and mushroom species on the studied traits. The oyster mushrooms exposed to low temperature had a significant effect on the carpophore area and thickness. Moreover, there was a significant difference between the carpophore areas of two oyster mushroom species at low temperature and between the carpophore thicknesses of two oyster mushroom species at control temperature. The effect of low temperature on the change in K content was found to be significant in both oyster mushrooms. Gray oyster mushroom had significantly higher (17.54%) K content than the yellow oyster mushroom at control temperature, while yellow oyster mushroom had significantly lower (17.49%) Mg content at low temperature compared to control temperature. In low temperature environment condition, the CAT activity increased by 5.7 times in gray oyster mushroom and 5.0 in yellow oyster mushroom. Moreover, The APX activities of mushrooms at low temperature increased by 8.1 times in gray oyster mushroom and 7.5 times in yellow oyster mushroom. Moreover, the yellow oyster mushroom had significantly (p<0.01) higher CAT and APX contents (0.57 and 1.43 times, respectively) than the gray oyster mushroom at low temperature.

Keywords: Low temperature, nutrient elements, Pleurotus ostreatus, P. cornucopiae var. citrinopileatus

1. Introduction

There are many types of mushrooms in nature. As a food for humans, mushrooms are a great source of protein and vitamins. However, since many of these mushrooms are poisonous, the number of people poisoned increasing. Cultivated every year is mushrooms are more preferred by people because they are non-toxic. Especially since oyster mushrooms grow in many environments such as bark, plant waste and bran, there is no trouble in preparing compost. It is cultivated in many regions due to its easy production and rich nutrient content. Pleurotus genus are commercially important edible mushrooms and are widely cultivated in both Türkiye and worldwide (İnci et al., 2022). After Agaricus bisporus, Pleurotus ostreatus (Jacq. ex Fr.) P. Kumm. (1871), also known as the oyster mushroom, is the edible fungus that is most widely cultivated globally. Pleurotus is a diverse category with a rising economic importance.

and Environmental growing media differences could have substantial effect on mushroom production. Regions with different climates can create abiotic stress on mushroom production. It is reported that abiotic stress negatively affects crop production (Yildirim et al., 2022). Studies conducted in regions with different climatic conditions provide important information about mushroom yield and quality. Among the mushroom samples pyragalus, belonging to Lactarius L. controversus and L. semisanguifluus species collected from the humid and cool Central Black Sea Region of Türkiye, L. pyragalus mushroom species is richer in terms of dry matter, ash, protein, N, Fe, Mg, Mn and K content (Pekşen et al., 2007). Four oyster mushroom species, Pleurotus ostreatus (grav). P. ostreatus (white), P. cornucopiae var. citrinopileatus (bright yellow) and Р. salmoneostramineus (pink) were studied in three media (100% wheat straw, 70% wheat straw, 20% white sawdust and 10% palm fiber, 50% wheat straw, 50% white sawdust) in a study examining the effects of, Co, Pb, Fe, Ni, Cu, Zn, Cd and Mn contents, and it was stated that there is no statistical difference in terms of mineral elements in all three environments (Owaid et al., 2015). In another study investigating the nutritional content of cultivated mushrooms; It was stated that the contents of N, P, K, Ca, Mg, Fe, Cu, Zn and Mn showed great significant differences due to the fact that they were purchased from different commercial enterprises and had different chemical compositions (Sönmez et al., 2016).

In the literature, there are several other studies reporting environmental, production and species differences among the mushrooms for the yield and nutritional aspects (Sher et al., 2010; Das et al., 2016; Garo and Girma, 2016; Wang et al., 2017; Bellettini et al., 2019; Krah et al., 2019; Kumar et al., 2020; Naim et al., 2020; Stojek et al., 2022; Cetin et al., 2023; Sheng et al., 2023). To make it easier to cultivate oyster mushrooms year-round, it is necessary to determine which Pleurotus species are suitable for the local climate conditions. Therefore, the present study was carried out in order to determine how low applied during temperature carpophore formation would affect growth and content of Pleurotus ostreatus (Jacq. ex Fr.) P. Kumm oyster mushroom), (1871)(gray Р. citrinopileatus Singer (yellow oyster mushroom). The aim of this study was to investigate the effects of low temperatures on carpophore area and thickness, potassium (K), calcium (Ca), and magnesium (Mg) contents, and the changes in catalase (CAT) and ascorbate peroxidase (APX) enzyme activities in these Pleurotus oyster mushroom species.

2. Materials and Methods

In the present study to determine the physiological development of produced in cool regions, two oyster mushrooms were employed: *Pleurotus ostreatus* (gray), *P. cornucopiae* var. *citrinopileatus* (yellow). The experiment was established with 2-liter ready-made mushroom production kits (90% oak shavings + 10% wheat bran) provided by Agroma company in Denizli province of Türkiye. The experiment was designed

according to the completely randomized experimental plot design with three replications each consisting four kits.

Mushroom kits are produced in the climate room having 55.67 luxury light intensity with a constant temperature at 22 °C. When the carpophore is fully formed, the half of the production kits were transferred another controlled production area with 10 °C and light intensity 54.33 luxury for low application for temperature 13-day development. Production kits at the control (22 °C) are also continued to produce without any changes in the climate room for 13-day development. At the end of the experiment, the samples from low temperature and control groups were examined for the analysis and measurements, carpophore area and thickness, potassium (K), calcium (Ca) and magnesium (Mg) content, catalase (CAT) and ascorbate peroxidase (APX) amounts.

2.1. Measurements and analyzes

2.1.1. Carpophore area

The carpophores of oyster mushroom samples were drawn on paper and their area were measured in cm^2 with a digital planimeter.

2.1.2. Carpophore thickness

The carpophore thickness of the oyster mushrooms was measured in mm with the caliper of the leafy part of the carpophore.

2.1.3. Mineral element analysis

Oyster mushroom samples taken from the kits in each mushroom environment were dried in 65 °C for 48 hours. Dried samples were burned until the ash in 550 the ash oven. The obtained ash samples were filtered on a blueband filter paper in HCL, then K, Ca and Mg readings were made on the atomic absorption device (AAS, Thermo Scientific, Model: iCE-3000 series) at the scientific research and application center of the Yüzüncü Yıl University. (Kabay, 2019; Kacar ve ark., 2006; Kuşvuran, 2010).

2.1.4. Catalase (CAT) activity

Catalase (CAT) activity was determined through monitoring the inhibition of H₂O₂ at 240 nm. As the reaction solution, 0.05 M phosphate buffer (KH₂PO₄) and 1.5 mM H₂O₂ mixture was used (pH: 7.0). About 2.5 ml reaction solution was mixed with 0.2 ml plant extract. Readings were performed in a spectrophotometer (Thermo Scientific, Model: GENESYS 10S UV-VIS) at 0th and 60th seconds. Reaction was initiated with the addition of 0.1 ml enzvme extract. Assessments were made by taking the change absorbance within a minute in into consideration (Jebara et al., 2005; Güneri Bagc1, 2010).

2.1.5. Ascorbate peroxidase (APX) activity

Ascorbate peroxidase (APX) activity was measured through ascorbic acid-induced inhibition of H₂O₂ at 290 nm. As the reaction solution, 50 mM phosphate buffer (KH₂PO₄), 0.5 mM ascorbic acid, 0.1 mM EDTA and 1.5 mM H₂O₂ mixture was used (pH: 7.0). About 3 ml reaction solution was mixed with 0.1 ml plant extract. Readings were performed in a spectrophotometer (Thermo Scientific, Model: GENESYS 10S UV-VIS) at 290 nm in 0th and 60th seconds. Reaction was initiated with 0.1 ml enzyme addition. Assessments were made by taking the changes in absorbance within a minute into consideration (Sairam et al., 2005).

2.1.6. Statistical analysis

The experiment was designed in a randomized plot design with two oyster mushrooms at two temperatures with four replications and 2-liter mushroom production kits in each replicate. Experimental results were evaluated statistically using the Independent sample t-test (Yesilova and Denizhan, 2016).

3. Results and Discussion

Oyster mushrooms (Pleurotus spp.) are widely cultivable and adaptable to a range of temperatures and various intrinsic and extrinsic factors affect their production (Bellettini et al., 2019). stated that due to its extraordinary ligninolytic characteristics, the *Pleurotus genus* is one of the white-rot fungus that has been studied the most; it is a mushroom that can be eaten, and because it contains significant bioactive compounds; it also has a number of biological effects; many common fermentation parameters, including medium composition, the carbon to nitrogen ratio, pH, temperature, air composition, etc., have an impact on the lignocellulolytic enzymes in basidiomycete fungus. Increase or temperature in mushroom decrease in production could affect yield and quality in mushrooms. In particular, ovster the determination of the effects of low temperature on yield and quality has been the subject of many studies. In the studies, it is emphasized that the yield and quality of the same varieties in cold and warm regions vary (Sher et al., 2010; Bellettini et al., 2019). Because the quality and yield values of mushrooms are suitable for market demand, which positively

affects production. For this reason, in the present study, nutrient content, carpophore measurements and enzyme activities in oyster mushrooms at low temperature were examined.

In the present study, it was found that oyster mushrooms exposed to low temperature had a significant (p<0.01) effect on the carpophore area and thickness. While there was a 16.16% decrease in the carpophore area of gray oyster mushroom (Pleurotus ostreatus) in the low temperature application, a 19.41% decrease was observed in the yellow oyster mushroom (P. cornucopiae var. citrinopileatus) (Table 1). On the other hand, carpophore thickness of the gray oyster mushroom decreased by 11.07% at low temperature and there was a decrease of 9.88% in yellow oyster mushroom (Table 1). Moreover, there was a significant (p<0.05)difference between the carpophore areas of oyster mushroom species at low two temperature and there was a significant difference (p<0.05) between the carpophore thickness of two oyster mushroom species at control temperature. Gray oyster mushroom had slightly larger and thicker carpophore compared to yellow oyster mushroom at low and control temperatures, respectively.

Table 1. Effects temperature on carpophore area and carpophore thickness in two oyster mushroom species

Oyster mushrooms	Carpophore area (cm ²) at 22 °C (control temperature)	Carpophore area (cm ²) at 10 °C (low temperature)	Change (%)	Carpophore thickness (mm) at 22 °C (control temperature)	Carpophore thickness at 10 °C (low temperature)	Change (%)
Pleurotus ostreatus (gray)	70.65 A a**	59.23 A* b	-16.16	2.62 A* a**	2.33 A b	-11.07
P. cornucopiae var. citrinopileatus (yellow)	69.97 A a**	56.39 B b	-19.41	2.53 B a**	2.28 A b	-9.88

*: There are significant differences (p<0.05) between the uppercase letters in the columns for two oyster mushroom species in control or low-temperature based on the independent sample t-test;

**: There are significant differences (p<0.01) between the vertical lowercase letters for the traits in control or low-temperature grown each oyster mushroom species based on the independent sample t-test.

The effect of low temperature on the change in K content was found to be significant (p<0.01) in both oyster mushrooms. At low temperature, K content decreased by 54.25% in gray oyster mushroom (*Pleurotus ostreatus*) and by 52.68% in yellow oyster mushroom (*P. cornucopiae* var. *citrinopileatus*) (Table 2).

Moreover, gray oyster mushroom had significantly (p<0.05) higher (17.54%) K content than the yellow oyster mushroom at control temperature (Table 2). Although the Ca content in the low temperature environment decreased by 15.45% in the gray mushroom, the Ca content in the yellow mushroom decreased by 20.00%, the effect of low temperature on the change in Ca content was found to be insignificant in both oyster mushrooms (Table 2). Moreover, yellow

oyster mushroom had significantly (p<0.05) lower (17.49%) Mg content at low temperature compared to control temperature (Table 2).

Table 2. Effects of temperature on potassium (K), calcium (Ca), and magnesium (Mg) contents in two oyster mushroom species

Oyster mushrooms	K content (%) at 22 °C (control temperature)	K content (%) at 10 °C (low temperature)	Change (%)	Ca content (%) at 22 °C (control temperature)	Ca content (%) at 10 °C (low temperature)	Change (%)	Mg content (%) at 22 °C (control temperature)	Mg content (%) at 10 °C (low temperature)	Change (%)
Pleurotus ostreatus (gray)	0.787 A* a**	0.360 A b	54.25	0.110 A a	0.093 A a	15.45	0.360 A a	0.307 A a	14.72
<i>P.cornucopiae</i> var. <i>citrinopileatus</i> (yellow)	0.670 B a**	0.317 A b	52.68	0.100 A a	0.080 A a	20.00	0.343 A a*	0.283 A b	17.49

*: There are significant differences (p<0.01 or p<0.05, respectively) between the uppercase letters in the columns for two oyster mushroom species in control or low-temperature based on the independent sample t-test;

**, *: There are significant differences (p<0.01 or p<0.05, respectively) between the vertical lowercase letters for the traits in control or lowtemperature grown each oyster mushroom species based on the independent sample t-test.

The change of catalase (CAT) and ascorbate peroxidase (APX) activities in both oyster mushroom species in low temperature environment was significant (p<0.01) compared to control temperature. In low temperature environment condition, the CAT activity increased by 5.7 times in gray mushrooms and 5.0 in yellow mushrooms (Table 3). Moreover, The APX activities of mushrooms at low temperature increased by 8.1 times in gray mushrooms and 7.5 times in yellow mushrooms (Table 3). Moreover, the yellow oyster mushroom had significantly (p<0.01) higher CAT and APX contents (0.57 and 1.43 times, respectively) than the gray oyster mushroom at low temperature (Table 2).

Table 3. Effects of tem	perature in CAT	and APX activities in two	o oyster mushro	oom species
	CAT content	CAT content	ADV content	APV content

Oyster mushrooms	CAT content (mmol g ⁻¹ FW) at 22 °C (control temperature)	CAT content (mmol g ⁻¹ FW) at 10 °C (low temperature)	Change (%)	APX content (mmol g ⁻¹ FW) at 22 °C (control temperature)	APX content (mmol g ⁻¹ FW) at 10 °C (low temperature)	Change (%)
Pleurotus ostreatus (gray)	8.755 A b**	56.698 B** a	570.45	3.144 A b**	28.762 B** a	814.21
P. cornucopiae var. citrinopileatus (yellow)	14.792 A b**	89.146 A a	502.66	8.186 A b**	69.900 A a	753.89

**: There are significant differences (p<0.01) between the uppercase letters in the columns for two oyster mushroom species in control or lowtemperature based on the independent sample t-test;

**: There are significant differences (p<0.01) between the vertical lowercase letters for the traits in control or low-temperature grown each oyster mushroom species based on the independent sample t-test.

Mushroom cultivation is an important aspect of agriculture, and the Pleurotus oyster mushroom species is widely cultivated due to its nutritional and medicinal values. Low temperatures have been reported to affect the yield and quality of mushrooms, and various studies have investigated the effects of environmental factors on the growth and yield of different mushroom species, including Pleurotus spp. In the literature, there are several studies reporting environmental and species differences among the mushrooms for the yield and nutritional aspects. Owaid et al. (2015) reported that there was no statistical difference in the C, N, Co, Pb, Fe, Ni, Cu, Zn, Cd and Mn contents of their carpophores of the oyster mushroom species, *Pleurotus ostreatus* (grey), P. ostreatus (white), P. cornucopiae var. citrinopileatus (bright yellow) and P. salmoneostramineus (pink). Sönmez et al.

(2016) stated that the N, P, K, Ca, Mg, Fe, Cu, Zn and Mn contents showed statistically large differences in the cultured mushrooms taken in enterprises where production place and temperatures are different. Pekşen et al. (2007) studied the mushroom samples belonging to Lactarius pyragalus, L. controversus and L. semisanguifluus species collected from the humid and cool Central Black Sea Region of Türkiye, L. pyragalus mushroom species in terms of dry matter, ash, protein, N, Fe, Mg, Mn and K content of other mushrooms and they stated that they had richer contents than the other species. Mycelial growth was observed in the medium consisting of a mixture of straw, sawdust and bran (45% straw + 45% sawdust + 10% bran) of four different Pleurotus ostreatus isolates (PO-141, PO-142, PO-143 and PO-144) obtained from different locations of Bolu province, and it was stated that the highest yield was obtained from PO-143 isolate in the study observing also first harvest time, mushroom weight, yield, cap length, cap width, stem length, stem diameter, color characteristics of the mushroom, pH, ash, dry matter, TSS and protein contents (Kibar, 2019).

Stojek et al. (2022) monitored mushroom production in the temperate mixed deciduous forest of Białowieża Primitive Forest in eastern Poland over two harvest seasons: Their research plots were established under similar environmental conditions (topography, geology, and soil type) but differed in tree species composition and tree species richness. At the end of their study, it was stated that higher precipitation in 2021 resulted in higher mushroom production compared to 2020, while lower precipitation levels in 2020 resulted in a stronger effect of ambient temperature (Stojek et al. 2022). In a study examining the properties contributing to yield and yield of oyster mushroom in Gamo Gofa in Ethiopia's low altitude Arba Minch (1278 m altitude) and high altitude Chencha (2741 m altitude) towns, it was reported that the yield was better in Chencha (Garo and Girma, 2016). In another study on the mushroom colors of the hot temperatures, it is stated that the mushrooms are darker at low temperatures

(Pleurotus ostreatus, P. florida, P. sajorcaju, P. ervngii. P. pulmonarius, P. citrinopileatus, P. flabellatus and P. fossulatus) were grown at 18 °C and 25 °C, and it was stated that while P. florida had the highest yield value at 18 °C, P. pulmonarius had the highest yield at higher temperature, while other species gave good yield at medium temperature (Das et al., 2016). Kumar et al. (2020) evaluated three oyster mushroom species (Pleurotus florida, P. eous, and Hypsizygus ulmarius) for the biological efficiency and net returns invested in two different seasons [late kharif (the monsoon) and rabi (the winter) seasons] in India. These researchers reported that biological efficiency and net returns invested of P. florida and H. ulmarius were on par and significantly superior to P. eous in both the seasons. Their study provided corroboration for the suitability of P. florida and H. ulmarius cultivation in the late kharif and rabi seasons, whereas P. eous cultivation in the summer and early kharif seasons; Thus, the season-specific selection of oyster mushroom species promotes the concept of year-round mushroom cultivation. Lower temperatures and dry condition could reduce stalk height and cap size of oyster mushroom. Sher et al. (2010) reported that in comparison to those growing in the Swat region in Pakistan, Peshawar region mushrooms had higher stalk height, stalk diameter, cap size, and fresh weight; In contrast, the Swat region had longer spawn running times, more fruiting bodies, and more productions of the studied mushroom than the Peshawar region did; The region of Peshawar's mild winters and Swat's cool summers were discovered to be ideal for *Pleurotus ostreatus* growth and production. In terms of the effects of low temperatures on carpophore area and thickness, Wang et al. (2017) investigated the differential expression patterns of *Pleurotus ostreatus* catalase genes during developmental stages and under heat stress. The authors reported that catalase genes were upregulated under heat stress, which indicated the importance of catalase in protecting the mushroom from environmental stressors. These researchers stated that catalases (CAT) are common enzymes that

(Krah et al., 2019). Eight oyster mushrooms

detoxify hydrogen peroxide; they aid in the growth and development of fungi, including mycelial growth and cellular differentiation, as well as the defense of fungi against oxidative damage under stressful situations, and their results indicated that studied two CAT genes may play different roles during development and under heat stress. Moreover, Gurgen et al. (2020) stated that cultivated *P. citrinopileatus* mushroom had a lower oxidative stress status and had antioxidant potential and this potential varied based on the substrate used.

Bellettini et al. (2019) studied the factors affecting Pleurotus spp. and reported that temperature, light, humidity, and substrate composition are important factors that affect the growth and yield of mushrooms. Cetin et al. (2023) investigated the effect of different production environments on yield and quality in mushroom (Pleurotus ostreatus) production and reported that temperature and humidity were the most important factors affecting mushroom yield and quality. Regarding the effects of low temperatures on mineral contents, Naim et al. (2020) investigated the variation of *Pleurotus ostreatus* performance subjected to different doses and timings of nano-urea. The authors reported that nano-urea treatment significantly increased the K, Ca, and Mg contents of the mushroom, which indicates the potential of nano-urea as a supplement for mushroom cultivation.

Finally, in terms of the effects of low temperatures on enzyme activities, Sheng et al. (2023) investigated the relationship between antioxidant enzymes and sclerotial formation of Pleurotus tuber-regium under abiotic stress. The authors reported that CAT and APX activities were positively correlated with sclerotial formation, indicating the importance of these enzymes in protecting the mushroom from stressors. Sheng at al., (2023) studied selationship between antioxidant enzymes [ascorbate peroxidase (APX), superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT)] and sclerotial formation of Pleurotus tuber-regium under abiotic stress and discovered that these antioxidant enzyme genes played an important role in the sclerotial formation under low temperature stress, and it is strongly suggested that antioxidant enzymes and abiotic stresses are closely related to sclerotial formation in *P. tuber-regium*.

4. Conclusion

The consumption of mushrooms, which are rich in nutrients and vitamins, has increased with the rise of epidemic diseases. In the present study, it was investigated how the yield and quality of mushroom production were affected, especially in places where cool and low temperatures prevail. Different mushroom species and low temperatures have an impact on the traits that were being researched. The area and thickness of the carpophore were significantly altered in oyster mushrooms when they were subjected to low temperatures. In addition, there was a substantial difference between the thicknesses and areas of the carpophores of two different oyster mushroom species at control and low temperatures. In both oyster mushrooms, it was discovered that low temperature had a substantial impact on the change in K content. At control temperature, the K content of gray oyster mushrooms was significantly greater (17.54%) than that of yellow oyster mushrooms, whereas the Mg content of yellow oyster mushrooms was significantly lower (17.49%) at low temperature than at control temperature. The CAT activity rose 5.7 times in gray mushrooms and 5.0 times in yellow mushrooms in low temperature environments. Moreover, the APX activities of mushrooms increased by 7.5 and 8.1 times, respectively, at low temperatures in both gray and yellow mushrooms. Moreover, the yellow oyster mushroom had considerably larger CAT and APX concentrations than the gray oyster mushroom at low temperature (0.57 and 1.43 times, respectively). The yield and quality at low temperatures do not affect considerably the market value in a way that will decrease. When the study and literature information are examined, we think that production can be made in particularly low temperatures, in regions where the average temperature does not fall below 5 °C or in controlled atmosphere conditions. The result of the study could

benefit scientific studies such as studies on oyster mushrooms. Low temperature conditions can have some significant effect on the growth and development of Pleurotus oyster mushrooms, as well as their nutritional and enzymatic properties. Specifically, lower temperatures can lead to smaller carpophores with thinner caps and lower K, Ca, and Mg contents. On the other hand, low temperature conditions can upregulate the activities of antioxidant enzymes such as CAT and APX, which may contribute to the mushrooms' increased antioxidant capacity. The increased expression of genes related to nutrient uptake and metabolism, as well as antioxidant defense, may play a role in the response of oyster mushrooms to Pleurotus low temperature conditions. In conclusion, the studied Pleurotus oyster mushroom species is somehow affected by low temperatures, which can lead to changes in carpophore area and thickness, mineral contents, and enzyme activities. Environmental factors, such as humidity, temperature, and substrate composition, play important roles in mushroom cultivation and vield. Further research is needed to fully understand the mechanisms underlying the effects of low temperatures on mushroom growth and yield, and to develop effective strategies for optimizing mushroom cultivation under different environmental conditions.

Declaration of Author Contributions

The authors declare that they have contributed equally to the article. All authors declare that they have seen/read and approved the final version of the article ready for publication.

Declaration of Conflicts of Interest

All authors declare that there is no conflict of interest related to this article.

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