FINAL THESIS

Hungarian University of Agriculture and Life Sciences

Buda Campus

Department of Vegetable and Mushroom Growing

Bachelor's

Black soldier fly larvae, *Hermatia illucens L.* effect on mycelium growth rate in *Agaricus bisporus* and *Pleurotus ostreatus*

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1. INTRODUCTION

Global mushroom production has exceeded 15.25 million tonnes. Fortune Business Insights (2021) suggests an increase to 24 million tonnes in 2028. Button mushrooms are the global favourites with over 40% of total sales. Shiitake constitute approximately over 25%, with oysters taking up 13% and other species taking up the balance (F.B Insights.2021). Mushroom production generates upwards of 45 billion USD annually as of 2005 and is expected to grow to 100 billion USD by 2030 (Chang S.T 2006). With such large monetization in the mushroom food industry, producers need to discover more efficient ways of cultivating mushrooms to reduce operational costs and maintain mushrooms as a feasible crop to tackle global nutrient requirements.

Aside from the monetary value of mushroom cultivation, adopting mushroom cultivation is one way of tackling global climate change, soil and water scarcity. With the increasing global population, our current food production systems are damaging the environment at an unprecedented rate. It is paramount to develop alternative processes to help remediate the past effects of our global food network. Development in the mushroom sector has the potential to alleviate poverty, hunger and national security through employment and social wellbeing (Jayaraman, S. et al 2024).

Farmers worldwide are facing more difficulties with planning and adapting to constant variances in climatic conditions. Maintaining yields to appease the demand pressure has forced workers to use excess fertiliser, pesticides and herbicides to combat the fluctuations in the environment. In Nepal, farmers are forced to adjust management policies to accommodate these changes that have greatly affect economic gain (Lamichhane, P. et al 2022). With mushroom cultivation, farmers can manage to operate in a closed environment, making it feasible for consistent production granting a more secure future for their operations. This allows farmers to reduce waste in farms through efficient processes, timely operation and without the drawbacks of environmental effects such as rain, strong winds that may damage crop (Beyer, D. M. (2022).

In the multitude of reasons why mushroom cultivation is important to our ecosystem; Mycoremediation is a study that has gained popularity with evidence suggesting that mushrooms can be used to help decontaminate soils from various hydrocarbons, heavy metals, pesticides, herbicides and cyanotoxins. The process of ameliorating soil can lead to reclaiming of land previously lost by pollution for agricultural purposes. Land reclamation can aid in providing farmers arable land in producing necessary yields for consumption. This provides investors reason to promote studies in mycology that have the potential in benefitting environmental conditions. Improving planetary growing conditions would increase popularity of mushrooms and symbiotically assist other global industries (Akhtar, N., & Mannan, M. A. 2021).

Lastly, regarding the benefits of mushroom cultivation, it is important to illuminate not only the economic and environmental benefits but the medicinal properties of mushroom species around the world. They can be utilised as health supplements and in combating global diseases due to the components found inside their cell structure. These will be discussed below when ascertaining the content of the mushroom species involved in this article.

Horticulture engineers around the world have attempted to cultivate mushrooms on various substrates (growing medium), using various technologies that aim to enhance growth conditions

Nutrient extracts are a basic step in manipulating and determining the ideal growth setting. Ideal growth means having the highest yield in the shortest time possible. In this experiment, we use Black soldier fly larvae that are largely produced as a protein additive in animal fodder. The production of these insects helps to process biological waste in a process known as entoremediation. The byproducts of insect frass can be used as organic fertilizer, whilst their exoskeletons can be used to acquire chitin and chitosan which can be used in medical science. Due to the reproduction habits of flies, manufacturers can produce large volumes in relatively confined spaces which make it an economically viable option. The nutrition additive serves as a catalyst in mushroom production reducing operational costs (Huseynli, L. et al 2023). Further research and developments are discussed in the Literature review.

1.2 Objectives

The project was setup to investigate and evaluate the effect of different concentrations of black soldier ant larvae protein on the mycelium growth rate of *Pleurotus ostreatus* and *Agaricus bisporus*. This would serve as a commercial product if addition of the protein benefits mushroom growth. Farmers reducing cultivation times serves as massive profit due to the reduction in expenses from wages and other overhead costs (Sergeyeva, N., & Vasilchenko, T. 2024). By increasing concentrations of black soldier ant larvae protein:

1. To determine if the effect positively correlates with enhanced mycelium growth rates in both *Pleurotus ostreatus* and *Agaricus bisporus*. The experiment will test 0%, 1%, 5%, and 10% protein concentrations in an agar medium.
2. To determine the prolific effect of protein in comparison to no protein at all when testing with only the water agar to determine if a protein source must be present in mushroom cultivation. This will help in understanding the relation of the carbon to nitrogen ratio that is dependent for mushroom yields.

Due to the inability to measure mycelium density, we cannot compare mycelium volume or content thus only measuring the diameter over a given time. This will give us the mycelium growth rate.

2. LITERATURE REVIEW:

2.1 Mushroom cultivation

The two species that will be experimented with are, *Agaricus bisporus* and *Pleurotus ostreatus*. Each species belongs to two different types of mushroom families. *Pleurotus* come from the family *Pleuroteaceae* which are saprotrophic fungi that are white rot wood decaying, meaning they can degrade lignin. This gives them the ability to degrade complex organic matter like straw, wood and bagasse (Rühl, Martin & Kües, Ursula. 2007). *Pleurotus* however are not able to degrade high concentrations of heavy metals such as copper and zinc that were found in the growing substrate, which led to colonization rates decreasing (Baldrian, P. et al. 2005).

Agaricus species, from the family *Agaricaceae*, order *Agaricales*, lack the same enzymatic activity therefore need to be grown on partially degraded material like straw, litter and manure (Atila, Funda et.al 2017). Approximately 420 species are currently recognized world-wide in the genus *Agaricus*. The genus contains toxic species such as *Agaricus xanthodermus* as well as several wild edible species, such as *A. campestris* (the field mushroom), and the species *A. bisporus* (the button mushroom), which has been cultivated for at least 300 years. (Burgess, M. M., & Cuthbertson, L. 2022)

Oyster mushrooms contain bioactive compounds such as polysaccharides, peptides, terpenoids, fatty acids, esters and polyphenols. The mycelium and fruiting bodies of *Pleurotus* species exhibit immunostimulatory, anti-neoplastic, anti-diabetic, anti-atherosclerotic, anti-inflammatory, antibacterial and anti-oxidative properties (Golak Siwulska et al 2018). *Pleurotus spp* have been experimented for their neurogenic properties, specifically *Pleurotus giganteus* and was found to be a dietary antioxidant, promoting neuronal health (Phan, C.W. et al. 2014).

Agaricus bisporus which is the most widely cultivated mushroom in the world contains bisporitin which may have potential bioactive properties. (Ragucci, Sara 2023). They contain carbohydrates, proteins, lipids, fibers, minerals, and vitamins. Medically, agaricus mushrooms contain active ingredients, such as polysaccharides, lipopolysaccharides, essential amino acids, peptides, glycoproteins, nucleosides, triterpenoids, lectins, fatty acids. Due to these active compounds, *Agaricus bisporus* have been reported to have antimicrobial, anticancer, antidiabetic, antihypercholesterolemic, antihypertensive, hepatoprotective and antioxidant activities (Atila, Funda et.al 2017). Figure 2 details the specific amino acids that are contained in button mushrooms for specificity.

Mushrooms contain a high amount of protein content with an average value of 23.80 g/100 g dry weight (DW). They normally demonstrate the complete essential amino acid profile suitable for the human diet

(Ayimbila, F., & Keawsompong, S. 2023). This makes them an important nutritional source globally. Coupled with the 100-billion-dollar industry, it is clear why improvements in mushroom cultivation are required.

Edible mushrooms, especially button mushrooms are a major source of nutrients. The table from Sharma, Kratika. (2018) demonstrates the nutritional content of *Agaricus bisporus*. Coupled on the side is the nutritional content of *Pleurotus ostreatus* mushrooms for a comparison (Lesa, K. N., et al 2022). All nutrients are subject to change if growing conditions are changed.

*Table 1 comparing Agaricus bisporus and Pleurotus ostreatus nutritional content per 100g (*Sharma, Kratika. (2018) and Lesa, K. N., et al (2022))

Nutrient	***Agaricus bisporus* (per 100g)**	***Pleurotus ostreatus* (per 100g)**
Energy	27 kcal	33 kcal
Water	92.45 g	89.0 g
Protein	2.5 g	3.31 g
Carbohydrates	4.1 g	6.09 g
Fat	0.1 g	0.41 g
Fiber	Not mentioned	2.3 g
Thiamine (Vit B_1)	0.1 mg (9% of RDA)	0.15 mg
Riboflavin (Vit B_2)	0.5 mg (42% of RDA)	0.4 mg
Niacin (Vit B_3)	3.6 mg (25% of RDA)	5.2 mg
Pantothenic Acid (B_5)	1.5 mg (30% of RDA)	1.3 mg
Vitamin C	0 mg	5.0 mg
Calcium	18 mg (2% of RDA)	4 mg
Phosphorus	120 mg (17% of RDA)	140 mg

Potassium	448 mg (10% of RDA)	420 mg
Sodium	6 mg	18 mg
Zinc	1.1 mg (12% of RDA)	0.7 mg
Vitamin D (D2 + D3)	0.2 µg	10 µg (Irradiated)
Sugar	1.98 g	Not mentioned

The amino acids contained in *Agaricus bisporus* are serine, leucine, proline, histidine, glutamine, isoleucine, cysteine, alanine, threonine, valine, tyrosine, aspartic acid, norleucine, arginine, lysine, and glycine. In comparison, *Pleurotus ostreatus* contains only 13 amino acids. Methionine, phenylalaline, asparagine, hydroproxiline is only found in *Pleurotus ostreatus* and not *Agaricus bisporus*. *Agaricus bisporus* contains histidine, isoleucine, valine, trysonine, norleucine and arginine which is not found in *Pleurotus ostreatus* (Effiong, M. E et al 2024).

In appearance, *Pleurotus* mushrooms differ in many ways to the *Agaricus* species. Both grow as clusters but are visually very different. Figure 1 exhibits the difference in various mushroom families, including the differences in *Agaricus bisporus* and *Pleurotus ostreatus* mushrooms (refer to pic A and G in Figure 3) It also demonstrates growth habits, colour and size of the mushrooms and is used to demonstrate global diversity for contrast comparisons.

Figure 1 – Cultivated mushrooms worldwide - A. Agaricus bisporus, B. Auricularia auricula, C. Calocybe indica, D. Flammulina velutipes, E. Ganoderma lucidum, F. Lentinula edodes, G. Pleurotus ostreatus, H. Plerotus sajor-caju, and I. Volvariella volvacea. (Pala, Shaukat et al. 2014).

2.2 In vitro procedure

In vitro mushroom cultivation is a multi-stage process which requires careful administration. There are several culture conditions that affect mycelial growth. Nutrient media, carbon-nitrogen balance, temperature, humidity, pH levels, oxygen concentration, contamination control and light conditions (Bettoni, J. C. et al 2024).

The first step includes isolating a tissue sample of the chosen species. This can be any living, healthy part of the fruiting body. The sample is then sterilized, thermally or chemically. This can be done via wet heat sterilization known as autoclaving. Samples can be chemically sterilised using sodium hypochlorite at 1-10% concentration or using 70% ethanol. Antibiotics can also be added to inhibit bacterial growths that

would otherwise contaminate the tissue samples. (Thapa, R. et al 2017). The second step is to prepare a culture medium (nutrient media) for the tissue sample to propagate. Various mediums can be used containing sufficient sources of carbohydrates, nitrogen, vitamins, minerals and growth regulators (Bettoni, J. C et al 2024). Stanley and Nyenke (2011) found out that Malt Extract Agar (MEA), Corn Cob Extract Agar (CCEA) and Cassava Peelings Extract Agar (CPEA) media were found to stimulate luxuriant mycelial growth rate and extension whereas poor mycelial growth were recorded on Potato Dextrose Agar (PDA) and Plantain Peelings Extract Agar (PPEA) media.

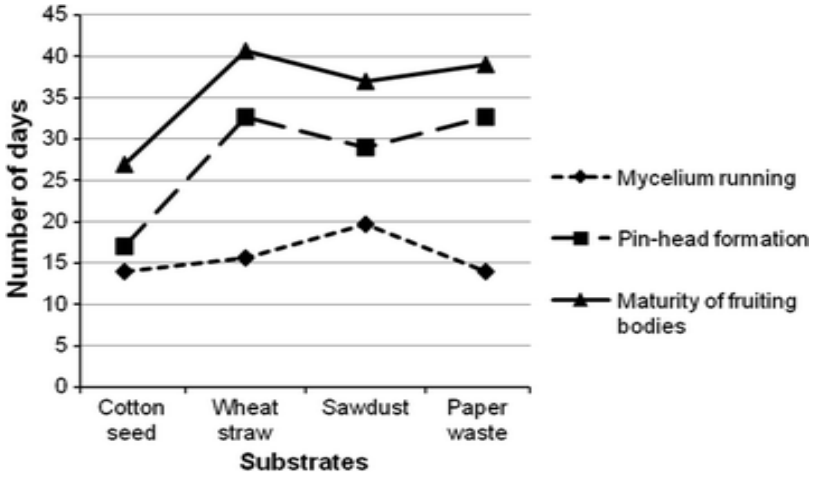
Once the medium is ready, it can be inoculated by the tissue sample and stored in incubation. The incubation period allows for the mycelium run until full colonization occurs. Sub-culturing is a procedure of transferring of microorganism into fresh nutritive medium from its stock culture (Jain, A. et al 2020). The mycelium is transferred to a fresh nutrient medium to maintain optimal growing conditions Once subculturing is done and the mycelium has spread through the new medium, the resulting product can then be transferred to the production area where production can be upscaled and controlled for the harvesting of the fruiting bodies (Jain, A. et al 2020).

2.3 Substrates and growing media

Current technologies depend on mycelial growth colonizing a substrate before pinning bodies are formed. These pin-heads then mature into fruits that are harvested which is the primary aim in mushroom cultivation. The mycelium run refers to the stage that the mycelium spreads and colonises a growing substrate (Suwannarach, N. et al 2022). The rate at which this mycelium will colonize greatly affects production time. Greater production time equates to greater costs which producers are looking to reduce.

In Figure 2, Girmay Z. et al (2016) tested oyster mushrooms (*Pleurotus ostreatus*) performance on different substrates and timed each growth stage. Cotton seed, wheat straw, sawdust and paper waste were used after the mycelium was propagated on a potato dextrose agar medium. It was evident that the substrate affects the mycelium growth rate shown by the number of days it took for the mycelium running to reach completion in each of the substrates. They analysed and charted out how long each process of the cultivation took from the mycelium run to pin head formation and finally to the mature fruiting body which is ready for harvest. Substrates with readily available nutrients performed more efficiently. From their experiment, they recommended to use cotton seed as the best substrate as it took the shortest time to complete all its stages. The mycelium running took 14 days, whilst the pin head formation took 2 more days and maturity completed at 26 days.

Figure 2 – Demonstrating time spent for different growth stages of Pleurotus mushrooms on different substrate (Girmay, Z. et al 2016)



In experiments carried out in Nigeria, using *Pleurotus* mycelium, a mixture of palm kernel cake and maize cob had the highest mycelia growth rate, sprouted 15 days after inoculation and yielded the highest total fresh weight of 2957.5 g). In comparison, a mixture of palm kernel cake and sawdust, sprouted 16 days after inoculation and yielded 2535.7 g fresh mushroom (Nwokoye, N. A. et al 2012). Lee Geon Six (2014) states that in *Agaricus bisporus* species, wheat grains showed fastest mycelial growth with 8.4 cm followed by rye, oat, barley with 8.2, 7.5 and 7.3 cm, respectively. Jatwa Tarun (2019) proves that for *Pleurotus florida*, sorghum had the fastest growth rate, but wheat showed the highest biological efficiency. This can be the case as mycelium growth can be affected by medium composition, ratio of carbon to nitrogen, pH, temperature and air composition (Bellettini, Ribani, R. H. 2019).

When dealing with fungal development, the carbon to nitrogen ratio is important. Carbohydrates serve as a carbon source, whilst protein serves as a nitrogen source. Substrates will commonly include materials that include both macromolecules which is why several experiments have been performed to assess the effect of different substrate materials in mycelial development (Hoa, Ha et al. 2015). As mentioned above, engineers have supplemented growing substrates with a multitude of additives to observe their effects. Each additive has benefits and drawbacks that need to be considered. For example, the black soldier fly that is used in this experiment has proven to be successful growth enhancer but contains an obnoxious odour, paired with flavour compounds such as trimethylamine, acetic acid, 3-methylbutanoic acid that has proven to cause flavour change in the yielded mushrooms (Huseynli, L. et al 2023).

Understanding mycelium's nutrient requirements help growers to enhance their production process. Ishaq, Muhammad (2017), discovered that in oyster production, out of 6 substrate mediums used for mycelial propagation; Potato Dextrose Agar (PDA), Potato Sucrose Agar (PSA), Malt Extract Agar (MEA), Corn Meal Agar (CMA), Yeast Extract Agar (YEA) and Water Agar (WA), the PDA performed the best with 11.0mm/day of growth. They also tested 9 different cereal grains, sorghum, wheat, chickpea, pigeon pea, pearl millet, soybean, maize, oats and beans as spawn for the propagation of the mycelium. From their results the sorghum performed the best with 10.66mm/day of growth. These are benchmarks that can be cost effective to growers by enhancing production yields and reducing production time.

Various supplements may also be added to growing mediums that will benefit mycelial growth. They are known to affect the nutritional values of the mushrooms whilst benefitting growth rates. Using rice bran bore different fat and potassium content in comparison to wheat straw (Salama et al., 2019). Producers have tried various products to improve nutritional content in mushroom development. Sawdust, corncob and sugar bagasse were tested to observe growth rate, yield and nutritional composition in *Pleurotus spp*. What they discovered was the carbon/nitrogen ratio in all substrates had a direct correlation with colonization period, mushroom weight, yield and protein content of the mushrooms. Increasing corn cob and sugar bagasse proportions in the substrate led to higher concentrations of calcium, potassium, manganese, magnesium and zinc. It also led to an increase in cap diameter, stipe thickness, mushroom weight, yield, BE, protein, fibre and short stipe length (Hoa et al., 2015).

2.4 Environmental growth factors

Temperature can affect mycelium development in its rate of growth and its intensity(efficiency). Enzymatic activity in the mycelium is sensitive to temperature, therefore it is paramount to observe temperature changes in the environment. In testing temperature conditions for *Pleurotus spp*. Hu Y (2023) tested a range from 15 – 32°C and found the best temperature for mycelium growth was deemed as 22°C.

Another parameter that affects the growth rate of mycelium is the pH of the agar. When testing various species from the genus *Hericium*, some species performed better at a 4.5pH instead of a 5.5pH (Gonkhom, D., Luangharn, T., Hyde, K.D. et al. 2002). Information regarding pH levels differ from species to species. *Trichoderma reesei* seemed to propagate optimally at a pH of 4.8 (Hang, Z. X.2011). Research on *Agaricus bisporus* mushrooms states that a ph of 7 – 7.5 was ideal (Ma, Y.et al 2014). Regarding *Pleurotus* species, it was found that their mycelium thrives in a more acidic region of 5.5-6.5 (Zervakis, G. I., & Balis, C. 1996). The grower must create an environment in which the ideal pH is present for their growth objective.

Due to the nature of in-vitro technology, controlling humidity and oxygen are difficult as the petri dish is sealed, meaning it is in a closed system. Researchers state humidity levels of 85% - 95% are ideal for *Agaricus* and *Pleurotus* mycelium growth (Zervakis, G., et al 2001).

Fungi, thus mycelium differed in their response to modified atmospheres in biomass, ergosterol content, mycotoxin production and morphology (Silva, F. L. H., & Martins, M. L. 2005). Oxygen is necessary for respiratory functions in mycelium and essential for growth. Experiments were performed testing laccase activity, glucose and biomass content in *Pleurotus* species using 3 different samples exposed to varying concentrations of oxygen (Uncontrolled, 30% and 80%). The results showed that the fungi performed best at 30% concentration (Bettin, F., Rosa, et al 2020). These results corelate with Mao, X.-B., & Zhong, J.-J. (2004), that tested oxygen concentrations of *Cordyceps militaris* and found low levels not supporting growth of mycelium, while too high a concentration also caused a detrimental effect. It was discovered that 30% was the best oxygen concentration when compared to 10% and 80%. Other parameters such as carbon dioxide have similar effects, in that there is a peak concentration in which the mycelium benefits (Dong, Y. et al 2018).

Mycelium can also benefit from other elements present in the growing medium. Nitrogen is essential for metabolic functions and was documented to have a beneficial effect at the right amount. The addition of nitrogen increased plant growth but decreased mycorrhizal fungal abundance. Elements such as phosphorous was also tested and in high concentrations increased total plant biomass and extraradical hyphal length, but shoot biomass largely increased in low phosphorus conditions (Dong, Y. et al 2018).

Other contributing culture conditions involved have their respective effects on mycelial growth rate. With in-vitro cultivation, mycelium runs do not require light. It is only at the fruiting body stage where mushrooms require light which affects their physiological and chemical structure (Stuper-Szablewska, K. 2022). Gao, R. et al (2018) differs in this information stating that dark, white, red, yellow, green, blue and purple light at different wavelengths had numerous effects on not only the production of hyphae, but their composition. Poyedinok, N. et al (2015) stated that low intensity coherent blue light was optimal for growth, melanin synthesis and all cellular activity.

As a process, in vitro technology requires strict controls and precise accuracy to gain the best results. With many variables to monitor, it is advised to be done in strict laboratory conditions. This coupled with professional equipment and a skilled workforce will make the process manageable.

2.5 Microbial Contamination

If propagation of mycelium is ideal then no infection will occur during the process, but all propagation methods have challenges. Microbial infections during mushroom propagation can have significant effects on the health and productivity of mushroom crops. These infections are often caused by pathogenic fungi, bacteria, or even pests such as nematodes and mushroom flies, which can lead to diseases that disrupt the normal growth and development of mushrooms (Preston, G. M. et al 2019). Microbial infection is a likely occurrence with in-vitro mushroom cultivation. Fungal contaminants such as *Aspergillus spp, Penicillium spp* and *Trichoderma spp* are commonly encountered. *Bacillus spp* were also commonly found (Gupta, Sachin et al. 2020). *Trichoderma pleurotus* and *G. penicillioidesis* were found to be the leading cause of green mould in oyster propagation (Ponnusamy, A., et al 2022).

There are various methods used to control contamination in mycelium production. Autoclaves are used in sterilizing apparatus used in fungi propagation (Singh, Raghuveer & Kangjam, Valenta. 2019). This is due to their high pressure and temperature making it sterile. Selective antibiotics such as streptomycin or penicillin may be added to the growing medium mixture to inhibit bacterial growth. Loefer, J. B., Bieberdorf, F. W., & Weichlein, R. G. (1952) stated that only large amounts of 3.5 - 8milligrams per millilitre were seen to cause disruption in fungal development, but doses below this only affected bacterial growth.

Preston G.M et al (2019) describes the use of microbial agents like compost teas to control pathogens by introducing beneficial microbes that can outcompete or inhibit their growth. Special care must be taken in the treatment of these teas as some teas can be contaminated before use.

3. MATERIALS AND METHODS

3.1 Materials Required

Apparatus Required for the experiment: Refer to Figure 3, 4, 5 and 6.

- 25 petri dishes for replication
- Parafilm for sealing petri dishes
- Sterile chamber
- *Hermetia illucens L.* protein source
- Potato Dextrose Agar
- Water agar
- Autoclave
- Distilled water
- Weighing scale
- Measuring cylinders
- Tissue culture of *Agaricus bisporus* and *Pleurotus ostreatus*
- Rubbing alcohol for disinfecting surfaces and tools

3.2 Methodology

Firstly, the sterile chamber is cleaned, and all procedures are carried out in the chamber to avoid contamination.

Four petri dishes contained Potato dextrose agar and concentrations of 0% protein content, 1%, 5%, and 10% with one petri dish containing only water agar and 10% protein content. This is the control to evaluate against the other trials and determine if mycelium can be cultivated only with protein. This made a total of 5 different petri dishes with differing constituents. Each petri dish contained 20ml of mixed/prepared agar. These petri dishes were replicated five times to be able to create a statistical average. The petri dishes were labelled according to their concentrations and content. For example, Potato dextrose agar with 1% protein concentration was named PDA1. Thus, there was PDA0, PDA1, PDA5, PDA10 and WA10.

To prepare the PDA and the protein constituents Dissolve 39g of PDA powder in 1L of distilled water. This mixture was separated into 4 different beakers each containing 250ml of PDA liquid. Protein percentages were measured as follows: 1% protein: Add 10g protein, which equated to 2.5g for 250ml. 5% protein: Add 50g protein which equates to 12.5g for 250ml. 10% protein: Add 100g protein which equates to 25g for 250ml of PDA mix. The water agar mix was done similarly, just using water agar instead of PDA.

Each of the ingredients was measured using precise digital scales and repeated to provide accuracy. Once the components were measured, they were placed in their respective beakers for mixing.

The respective beakers were then placed in an autoclave to sterilize, deaerate, and activate the agar. This is done by the autoclave using high temperatures of 121°C and a pressure of 15psi to achieve sterilization. The autoclave is run for 20 minutes as the volumes in the petri dish are not high and this is sufficient time to kill microbial activity.

After autoclaving the mixture is left to settle and cool down in the sterile chamber to 50 – 60°C (safe handling temperature). Once this is complete, the tissue culture from the appropriate mushroom species is placed in the centre of the petri dish for equal growth distribution.

Once completed, the mixtures containing the tissue culture sample were placed in storage at 25°C for the duration of the experiment. Humidity and light were negligent in this experiment, as the Petri dishes were airtight and leakproof.

Each petri dish was given one week before growth measurements were taken to allow the mycelium to establish itself from the tissue culture.

Diameter measurements were taken daily at a specified time, 0900hrs to avoid discrepancies in growth rates that may arise with lack of consistency. Using a vernier calliper, sensitive to one-hundredth of a millimetre for accurate measurement, the hyphae maximum extension length was recorded. An opaque background is used when measuring mycelium growth for visible clarity through the agar medium as it may be difficult to see their tiny hyphal projections otherwise. After one week of measurements, the experiment is terminated for the *Pleurotus ostreatus* mycelium and the results are analysed. The *Agaricus bisporus* takes longer and therefore is run for 22 days before termination and results analysed.

Once the experiment was completed, the results were compiled in Microsoft Excel for analysis. The results were tabulated for statistical awareness and graphs were drawn out to visualize key data points. The ANOVA statistical analysis was run to determine if the results were significant and coherent with our objectives.

All experiments were carried out in the Mushroom laboratory of the Department of Vegetable and Mushroom Growing in the Hungarian University of Agriculture and Life Sciences.

Figure 3 – Sterilizing equipment used in the experiment (Clockwise from top: Rubbing alcohol, autoclave, sterile chamber)

Figure 4 – Petri dish and parafilm used to seal petri dish

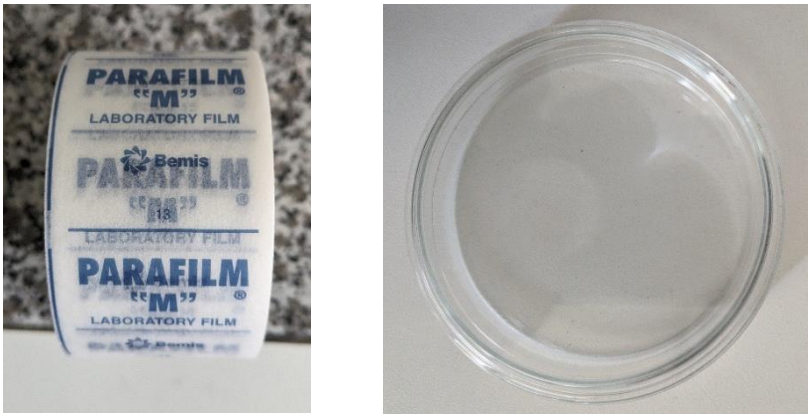


Figure 5 – Additives for growing medium (Water agar and Potato Dextrose agar)

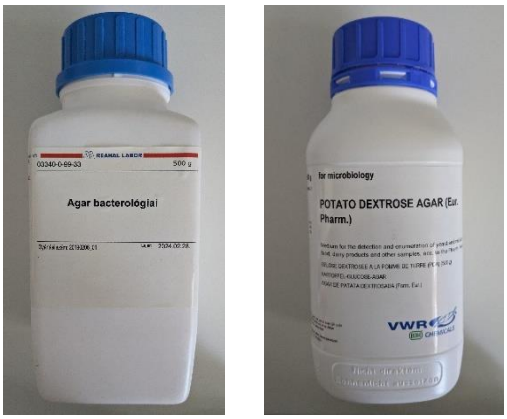
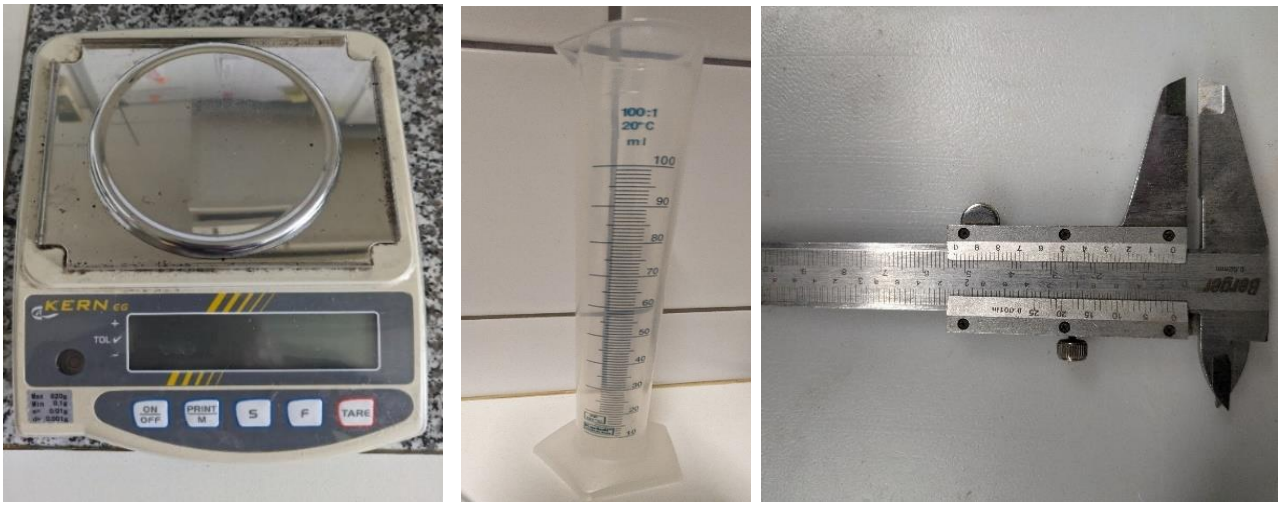


Figure 6 – Measuring apparatus for the experiment



4. RESULTS

4.1 *Agaricus* Results

Figure 7 - Graph showing the growth rate of Agaricus mycelium in different growing mediums; PDA0%, PDA1%, PDA5%, PDA10%, and WA10%. Measurements were taken on specified dates noted using a vernier caliper for precise millimeter readings. Key data has been labeled

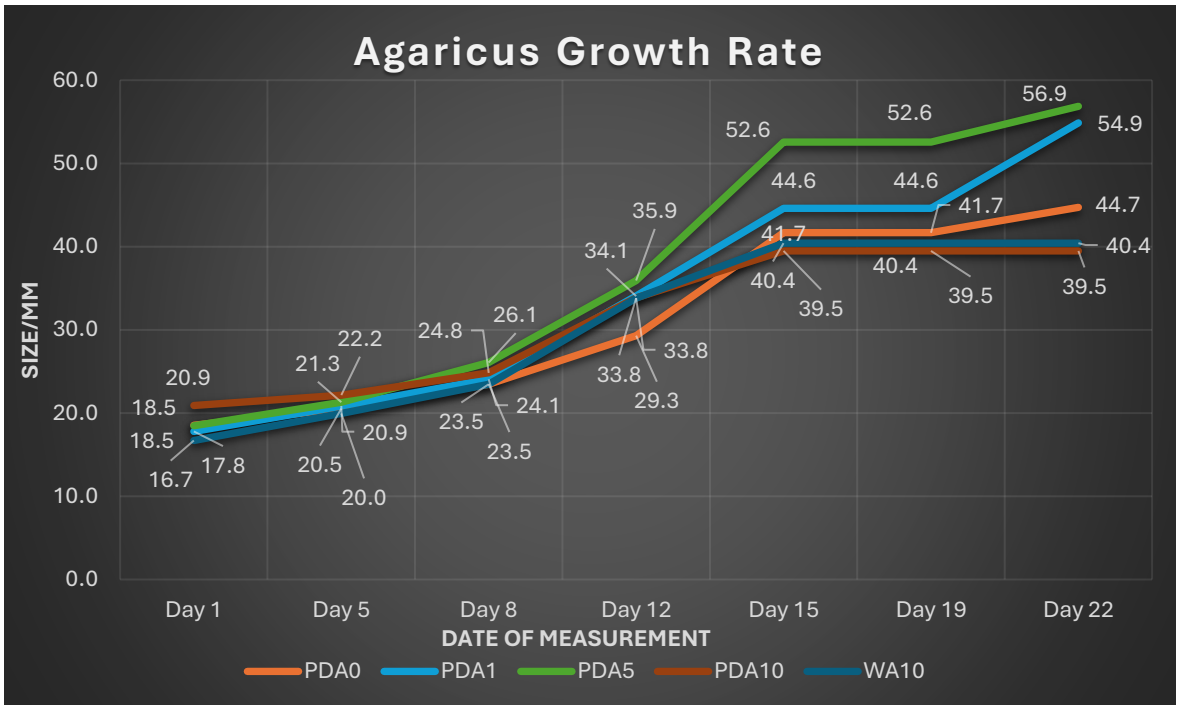


Figure 7 demonstrates graph values in millimetres of *Agaricus* mycelium diameter size. The raw data was immense, so each sample and repetition were averaged to create single-line graphs for each condition that is displayed above.

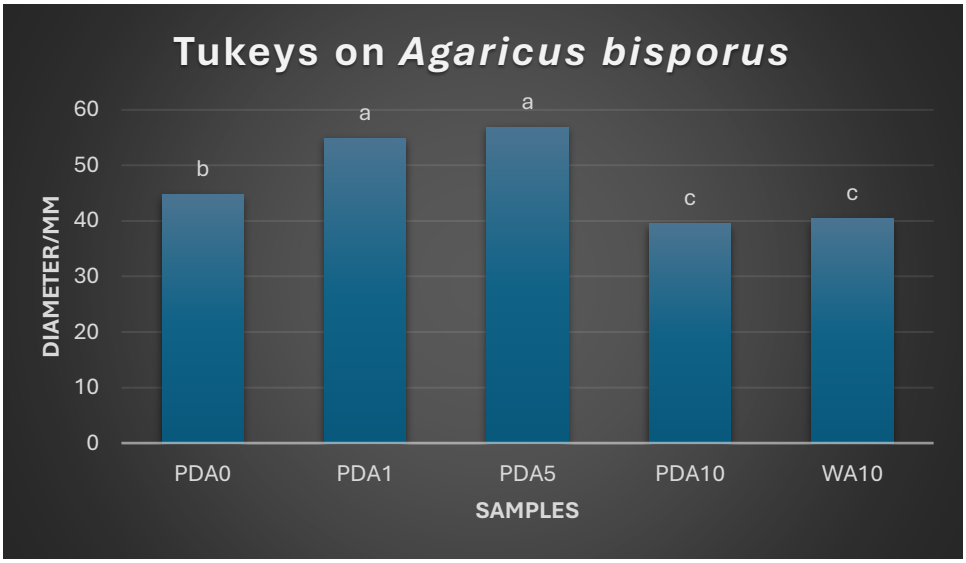
From the graph above, the PDA5 percent performed the most efficiently. It reached the largest diameter in the given time allocated and grew at the fastest rate according to the gradient of the line.

Table 2 demonstrates ANOVA statistical analysis for measurements taken. Key information in bold demonstrates a P-value less than 0.05.

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	2843.032	4	710.758	4.850413	**0.000783**	2.393438
Within Groups	60812.26	415	146.5356			

The p-value showing that it is less than 0.05 supports that a protein supplement will affect the growth rate, which is apparent.

Figure 8 - Graph showing Tukey analysis on the samples.



Agaricus mycelium is slower to colonize, therefore results were taken at longer periods and the results were collected over the space of 21 days.

5.2 *Pleurotus* results

Figure 9 - Graph showing the growth rate of Pleurotus mycelium in different growing mediums; PDA0%, PDA1%, PDA5%, PDA10%, and WA10%. Measurements were taken on specified dates noted using a vernier calliper for precise millimetre readings. Key data has been labelled on the graph.

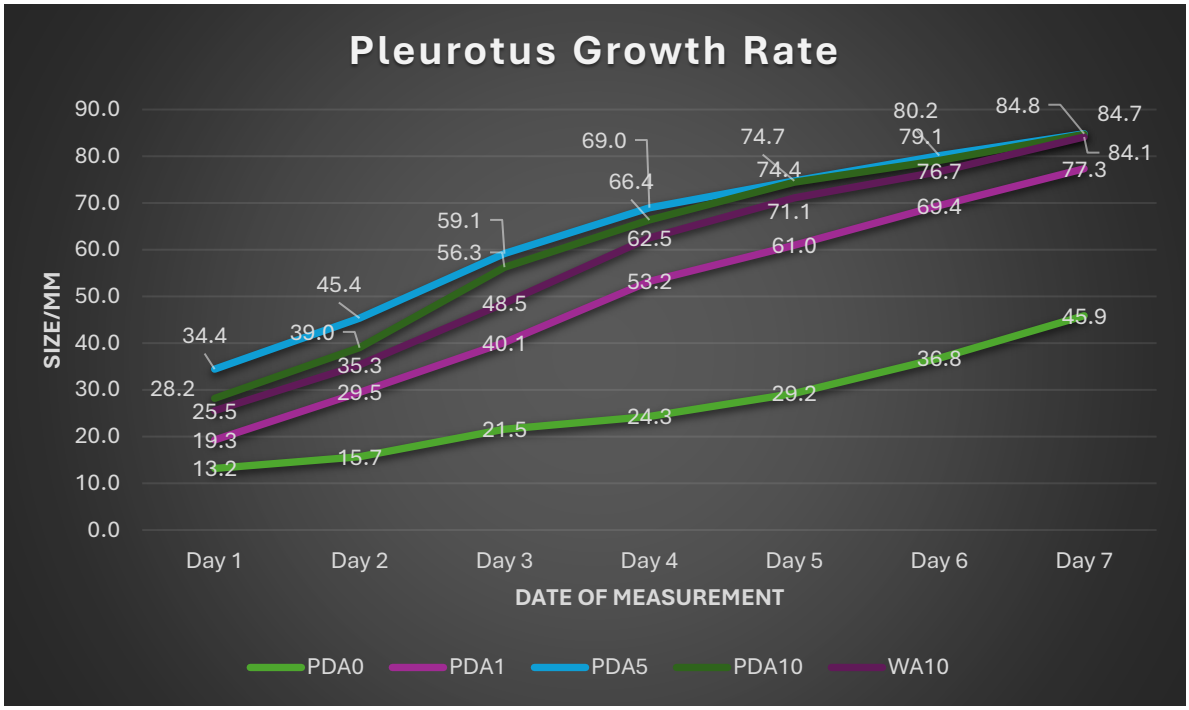


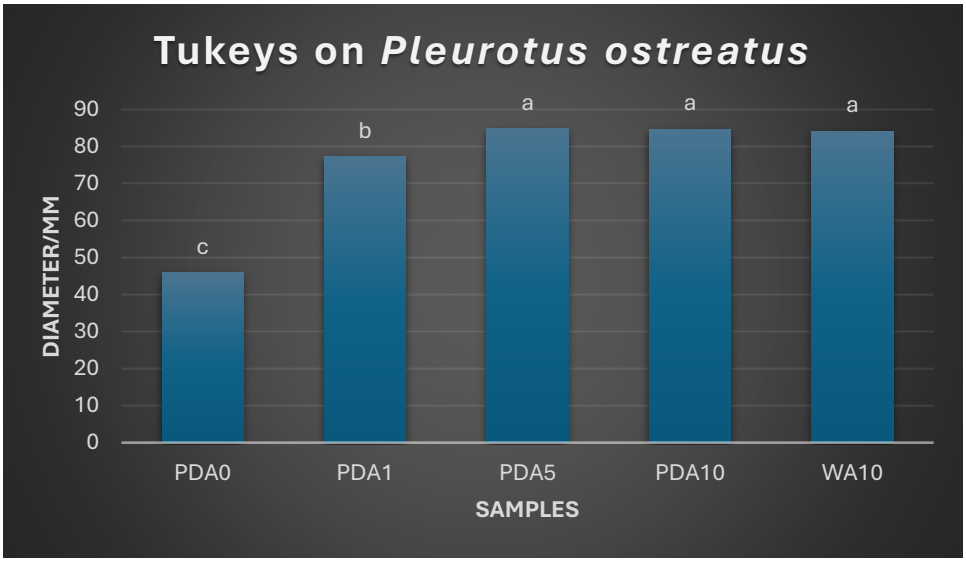
Figure 9 demonstrates graph values in millimetres of *Pleurotus* mycelium diameter size. The raw data was immense, so each sample and repetition were averaged to create single line graphs for each condition that is displayed above.

Table 3 shows results from ANOVA statistical analysis.

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	76051.08	4	19012.77	57.37103	**1.6138E-38**	2.393438
Within Groups	137531.1	415	331.4002			

From the ANOVA single factor analysis, the p-value determined was less than 0.05, statistically proving that the protein does have a direct impact on growth rate.

Figure 10 – Graph showing Tukey analysis on the samples.



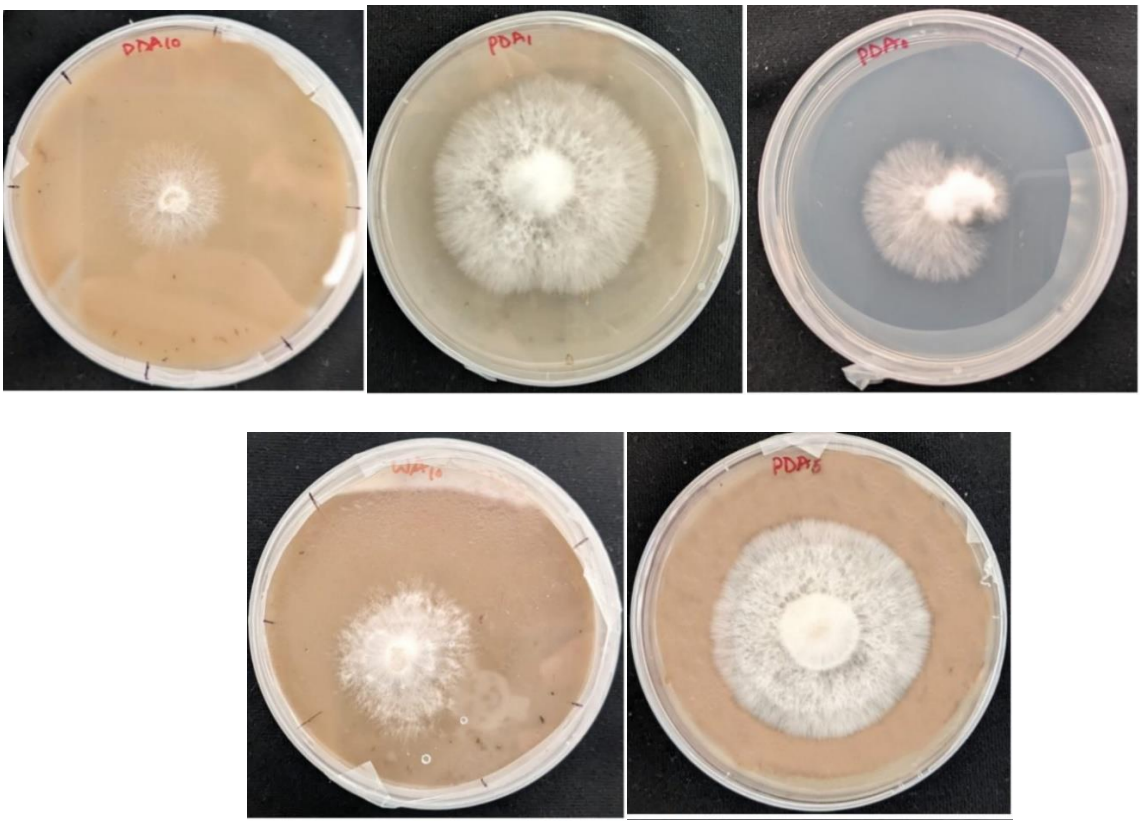
The results show the effect of 5 samples that had different growth supplements used for the propagation of mycelium from a tissue sample.

From the results given, a protein supplement enhances mycelium growth. According to the gradient of growth rates, there also is a threshold in which adding protein has no beneficial effect. This threshold occurs after a 5% protein dose using Potato Dextrose Agar (PDA).

In all cases though, a protein supplement does enhance the growth rate of mycelium.

The results also show that if PDA is not present, a water agar, with no glucose supplement can serve as a growing medium. According to the results, the protein supplement by itself in the water agar medium serves as a compatible growing supplement and has a more beneficial growth effect than a medium with no protein at all.

Figure 11 - showing different stages of growth of Pleurotus ostreatus in the 5 samples. Patterns and diameter differ in each sample. Labels in red on each sample



5. CONCLUSION

5.1 Results Discussion

Based on the results acquired, the tests were run with identical environmental conditions, except for the protein content, it can be concluded that the *Hermatia illucens L* protein powder additive, had a positive effect on mycelium growth. According to the objectives of this research, we found that the protein played a significant role in mycelial development. The results did show that the effect was not beneficial after a specific threshold of five percent, meaning there was a point that adding more protein did not beneficially affect the growth rate.

In *Agaricus* mushrooms, the mycelia with a 5% protein additive (PDA5) exhibited the maximum diameter between all the samples in 22 days with 56.9mm whilst the 10% (PDA10) had the minimum with a diameter of 39.5mm. With these results, it is conclusive to state that adding more protein is not beneficial to *Agaricus* mushrooms and a limit should be introduced of 5%. Another observation is that the WA10 grew slower relative to the other samples, showing that the *Agaricus* species are more sensitive to the carbon-nitrogen ratio, and thus require a source of carbohydrates to enhance growth. PDA 1 and PDA0 were the second and third-best trials, also showing that farmers should avoid adding high levels of protein when trying to propagate Agaricus mushrooms. After running a Tukey analysis on the sample data (Figure 8), it revealed that PDA1 and PDA5 did not have a significant difference and could be considered viable options. Farmers can opt to use 1% or 5% due to the insignificance of the test.

The maximum diameter that the *Pleurotus* mycelia reached over the 7 days was 84.8mm with a 5% protein additive (PDA5). In contrast, the lowest diameter reached was 45.9mm with no protein present (PDA0), which shows the importance of protein content in the agar medium. All results with the *Pleurotus* mycelia showed an increase in growth with protein additives. The diameter range between PDA1, PDA 10, WA10 and PDA 5 lay between 77.3mm and 84.8mm, showing a difference of 7.5mm between the trials. It can thus be suggested that farmers should ideally use 5% concentration. Adding 10% protein is an extra cost that farmers should avoid. After running a Tukey analysis on the sample data (Figure 10), it revealed that the differences in PDA5, PDA10 and WA10 are insignificant and could be considered as viable options. Farmers can opt to use either treatment but would be incurring extra costs on the protein supplement if using more additives.

Another objective was that the protein effect would be substantial as opposed to having no protein at all. From the results, the sample containing only PDA and no protein at all performed worse than the water agar

with 10% showing that protein content has a prolific effect on mycelium growth. Concerning the carbon-to-nitrogen ratio, the results revealed that a high nitrogen quantity is preferred over carbon.

The results correlate with other scientific studies that tested the effect of various supplements from common foods on mycelium growth. Proteins can benefit the growth of mycelium in controlled doses. (Soh E, Saeidi N, Javadian A, Hebel DE, Le Ferrand H 2021).

The patterns illustrated in mycelium growth observed are examples of the ways mycelium may express itself. Not only the diameter but the forms that the mycelium takes in the petri dish are affected by the protein (Fig 11). This is supported by Wang J. et al. (2023) that different amino acids have a diverse effect on mycelial growth patterns. Further studies in this field would involve determining the exact protein content in the additive to visualize each protein's effect and what benefits each has. Protein isolation is a 5-step procedure that results in a specific protein and would be the method used to carry out this experiment (Lee, C.-H. 2017). One recommendation would be to determine the actual protein involved in the experiment as discussed in previous research, different proteins have different effects on mycelium growth and can be a large factor when deciding quantities of components that can be added to a growing medium (Soh E, Saeidi N, Javadian A, Hebel DE, Le Ferrand H 2021).

Noted that at first, this was not an objective we set out with, but due to the results obtained was a notable occurrence.

5.2 Recommendations

Testing more parameters such as temperature and pH, will give more comprehensive results as commercial growers may not be able to keep environmental conditions as consistent as in a laboratory and these effects should be noted for a broader scope. Measuring the weight of the mycelium mass would also be another parameter that would help detect any changes in the mass as mycelium develops. With the utilization of the carbon and nitrogen sources, there could be changes in the total mass that will help determine if secondary processes are occurring. Schoder, K. et al (2024) discuss the idea of changing growth habits based on the depletion of nutrients and the conjuncture of changing pH and microclimatic conditions of the hyphae mass. Due to the mycelium using the available nutrients in the petri dish, there will also be an increase in growth rate in the early days of the project, with a gradual decrease in growth rate due to their consumption. Analysing these effects over a longer time will draw out more precise and accurate results regarding the effect of growth conditions.

In this experiment, we only tested the radial growth rate as a parameter to determine the effect of black soldier fly larvae protein. There are various other parameters to analyse when creating a defined and comprehensive overview of the effects on mycelium growth. Physical parameters include hyphal density, width, branching, pattern, colour, and mycelial biomass. Other parameters also involve biochemical analysis such as enzyme activity, lipid content, gene expression, and odour. The biochemical parameters involve more complex processes such as chromatography and spectrometry.

6. SUMMARY

Mushroom cultivation is important to support global food production. A billion-dollar industry requires novel ideas to escalate efficiency and meet the demands of a rapidly growing food technology (El-Ramady, H., et al 2022). The main objective of the experiment is to determine protein efficacy in mycelium development, producing feasible results to implement on a commercial level. We set out to use *Hermatia illucens L*, which is black soldier fly larvae that is used as a protein source for animal feed. Being capable of converting waste and manure into insect biomass, they can subsequently be used as a nutrition source for mycelium and mushroom production (Wang, Y.-S., & Shelomi, M. 2017). Using different concentrations of this protein source, we set out to determine the effect of adding it to mycelium growth substrate. With the results, we determined that commercial mushroom producers can boost production of *Agaricus bisporus* and *Pleurotus ostreatus* mushrooms and reduce costs in production. This is achieved by adding 5% black soldier fly larvae in the growing substrate which increases the growth rate of mycelium, reducing time and overhead costs of production.

In conclusion, the results determined that adding a protein source is beneficial for mycelium growth. It can be advised that growers use 5% protein additive to their substrate mixtures.

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8. LIST OF TABLES

9. LIST OF FIGURES

10. DECLARATIONS

DECLARATION

the public access and authenticity of the thesis

Student's name:	Daniel Andrew D'Souza
Student's Neptun code:	HRPJ40
Title of thesis:	Black soldier fly larvae, Hermatia illucens L. effect on mycelium growth rate in *Pleurotus ostreatus* and *Agaricus bisporus*
Year of publication:	2024
Name of the consultant's institute:	Hungarian University Of Agriculture and Life Science
Name of consultant's deparment:	Mushroom and vegetable growing

I declare that the final thesis submitted by me is an individual, original work of my own intellectual creation. I have clearly indicated the parts of my thesis or dissertation which I have taken from other authors' work and have included them in the bibliography.

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STATEMENT ON CONSULTATION PRACTICES

As a supervisor of DANIEL ANDREW D'SOUZA - HRPJ40, I here declare that the thesis has been reviewed by me, and the student was informed about the requirements of literary sources management and its legal and ethical rules.

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