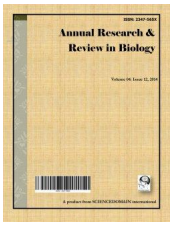

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Analysis of Physicochemical Parameters to Evaluate the Mycelia Growth of *Pleurotus pulmonarius*

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Authors' contributions

This work was carried out in collaboration between both authors. Author NI designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author FAA managed the analyses and the literature searches of the study and edited the manuscript. Both authors read and approved the final manuscript.

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ABSTRACT

Pleurotus pulmonarius commonly known as the grey oyster mushroom is the most widely cultivated species for its nutritional, medicinal and economical values. Two strains (China & Thailand) of *P. pulmonarius* were grown in potato dextrose agar (PDA) and sawdust substrate in different salinity conditions with different water collected from four resources to analyse the vegetative growth of mycelium. The water samples were labelled as W1-lab water (control), W2- Nas-Agro farm water, W3- Ganofarm water and W4- 7-star agricultural farm water. The present research evidently indicated that the two strains of *P. pulmonarius* showed relative similarities regarding their development and physiochemical characteristics. The mycelia of *P. pulmonarius* showed considerable growth for 0% and 1% NaCl in both PDA and substrate medium. The slight growth for 5% NaCl was observed only in sawdust substrate for W2 (water collected from Nas-Agro Farm) while remaining 10% NaCl plates (PDA) and test tubes (sawdust substrate) remained uncolonised. Among the NaCl concentrations tested, the best mycelia yield was recorded in the medium with low concentrations of NaCl as an essential element for the mycelial growth. Variation of mycelial growth was also observed with different water samples, growth medium and strains of the mushroom. The

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best results were observed in W2 with PDA media showing high vegetative growth. Moreover, the China strain showed faster and uniform results. This suggests that salinity, growth medium and water are effective for the growth of mycelium.

Keywords: Oyster mushroom; breeding; salinity; pH; farm water.

1. INTRODUCTION

Effective and continuous researches are required for the advancement of edible mushroom cultivation. The genus *Pleurotus* comprises popular edible mushrooms for their nutritional and medicinal properties, vigorous growth and cost-effective cultivation [1].

Pleurotus pulmonarius commonly known as the grey oyster mushroom is a species of *Pleurotus* which has become the second most widely cultivated mushroom universally. Grey oyster mushroom is an essential nutritive mushroom with high protein content and medicinal properties such as anti-tumor, anti-inflammatory, anti-microbial and immuno-enhancing [2].

Cultivation of the oyster mushroom has been increased greatly throughout the world during the last few decades. Many different techniques and substrates have been successfully utilised for mushroom cultivation and biomass production. Their favourable properties have given them an important place among the commercialised basidiomycetes [3]. In recent years, continuous research has been done on the development of edible mushroom strains that will increase the yields in term of fruit body and other bio-chemical substances [4].

Hence, there is a need to increase the research studies on the quality parameters and other necessary properties depending upon the demand for mushrooms. The development of cost-efficient and the alternate substrate to culture oyster mushroom without surrendering mushroom quality is a major focus of many researchers and cultivators. It can be considered that the vegetative growth phase is much appropriate for the study as they give early results that could be manipulated accordingly to obtain best results. This would help the growers or the cultivators to nurture the mushrooms to acquire better yield of fruit bodies in mass production and for the higher economy [5].

To determine the primary development, performance of the mushroom, growth of *P. Pulmonarius* was carried out in in-vitro conditions. The fungal growth and cultivation of *Pleurotus* spp. were observed to show their best growth in saw dust substrate [6] in comparison to exposure to various other substrate in which they did grow but failed to give same results. Among agar media studied, Sabouraud's dextrose agar (SDA) and Potato dextrose agar (PDA) media were found most favoured for both soil and clinical isolates.

Previous research has been done on mushroom cultivation with different substrates by maintaining growth parameters such as temperature, relative humidity, water content, air, pH and light intensity for *Ganoderma lucidum* [7] which showed that light and pH are the most important factors for mycelial growth. The mycelial development was observed that depends upon other parameters such as pH, temperature, culture media and nutrient elements which were known to greatly manipulate the fungal growth in both field and laboratory conditions [8]. The combined factors of different water activity and temperature showed the difference in growth rate of fungi [9].

This study was conducted:

i. To analyse the vegetative growth of *P. Pulmonarius* under NaCl stress in PDA media and sawdust substrate.
ii. To obtain the best water sample from three different farms that show enhanced mycelial growth comparatively.
iii. To find out if there is any difference in the growth of mycelia for both the strains used.
iv. To compare the mycelial growth rate in PDA media and actual farm substrate in different physicochemical parameters (water and salinity).

Therefore, due to several advantages as mentioned previously, the selected mushroom *P. pulmonarius* is taken into the study to improve regular protocol for commercial growth and development within in vitro cultivations. This research work is carried out to contribute to the knowledge of how physicochemical parameters

such as water and salt effect the mycelial growth of *P. pulmonarius*. Also, the best conditions of mycelia growth of *P. pulmonarius* under different physicochemical stress with deferred vegetative growth in the medium are observed. This will serve to provide information about few parameters that would help to optimize the growth of the commercial mushroom cultivation in Malaysia.

2. MATERIALS AND METHODS

Experimental design: The fruit body of *P. pulmonarius* was obtained from three different Malaysian farms along with the water samples. The experiment was carried out in the Biotechnology, Microbiology and Pharmaceutical laboratories, Lincoln University College, Malaysia. Culturing was carried out with regular observation for growth under given parameters with three replications.

Sample collection or sources of materials: The spawn (fungal hyphae) specimen of two strains (Thailand and China) of *P. pulmonarius* were used for culturing. The water samples for the preparation of media and substrate were collected from three different farms in Malaysia, namely: GanoFarm Sdn. Bhd. in Tanjung Sepat, NAS Agro Farm in Sepang and 7 Star Agricultural Sdn Bhd in Semenyih Jaya along with other ingredients like sawdust (Rubber Wood), rice bran and $CaCO_3$. The other equipment, consumables such as; test tubes, Petri plates, inoculation loop, NaCl, and PDA were provided by the university. Distilled water from the university laboratory was used as a control.

Culture technique: A simple procedure of tearing the fruiting body was developed for extracting the internal tissue and culturing it on media (test tubes or Petri plates).Vigorous mycelial growth was obtained from the young fruiting body using a scalpel dipped in 70% alcohol. Using a sterilized scalpel, a very small piece is cut from the clean inner tissue and then inoculated on to the surface of pre-prepared sterilized PDA plates with standard pH 5.6. The inoculated plates were subjected to incubation at standard room temperature in darkness inside the laboratory. PDA plates were colonized with beautiful white mycelium after 5-6 days as seen in Fig. 1. For further maintenance purification of the obtained cultures were done by repetitive sub-culturing. Pure culture was obtained for both strains (Thailand and China) of the mushroom. The best cultures among them were selected and stored as 'mother culture' from which further culturing was done in both Petri dishes (PDA) and test tubes (Sawdust substrate).

Stock culture: Stock cultures of the fungi collected were maintained in pre-sterilised Bijou bottles containing distilled water with 9.5 mm plugs taken from the pure culture plates. The cultures were reserved in the incubation chamber at 27°C for emergency and future use.

Fig. 1. Summary diagram of mushroom tissue culture technique

2.1 Preparation of Culturing Media

PDA media: Required amount of synthetic PDA mixture was mixed with four different samples of water containing four different salt concentrations which were autoclaved at standard temperature (121°C) and pressure (15 psi). The freshly prepared media was then poured into Petri plates after cooling down, then left to solidify under sterile conditions and then sealed with paraffin till inoculation of culture was done (Fig. 2).

Sawdust substrate: The general formulation for substrate preparation employed was 89% of sawdust, 10% of rice bran, and 1% of $CaCO_3$. The mixture was moistened with distilled water samples with different salt concentrations till the moisture content reached around 70% [10]. Close attention was paid to mixing for the even distribution of water throughout the entire substrate.

A palm test method is a simple way to check whether the mixture has the proper water content or not. A fistful of sawdust mixture is squeezed tightly if just a few drops of water gets released with pressure, the substrate mixture has the proper water content. If the sawdust is too wet, it will impede the free flow of air in the substrate which increases the chances of contamination.

Test tubes were filled and compressed with pre-weighed measurement (32.676 g) of sawdust substrate prepared equally, enclosed with cotton plugs (Fig. 3). They were then directed to standard sterilization in an autoclave for 1 hour to 30 minutes and cooled down overnight before culturing.

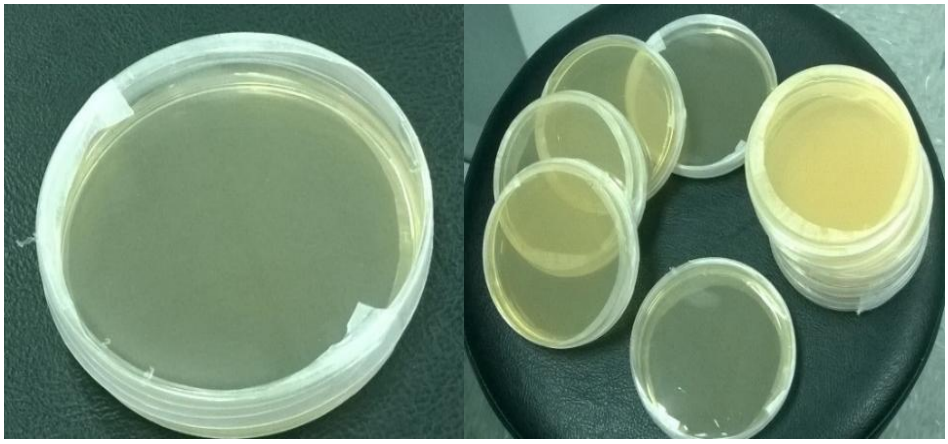

Fig. 2. PDA plates prepared

Fig. 3. Sawdust substrate filled test tubes

Attuning of salinity in water samples for mycelium growth: Firstly water from each four sample was divided into four parts with three parts having NaCl dissolved as per the percentage; 1%, 5% and 10% and one part with no salt taken as control. The pH before and after addition of $NaCl_2$ was recorded for further discussion. Four parts of water Sample were labelled as W1, W2, W3, and W4 with salinity percentages for each strain (e.g.: W1-0%, W2-1%, W3-5% and W4-10%). Each of these four parts was made for each water samples, then mixed with PDA and sawdust substrate composition.

2.2 Culturing and Incubation

Petri plates: Freshly prepared PDA plates were cultured with sub-culture plugs that were inoculated using a loop with a single plug (9.5 mm). Those were made using a cork borer, positioned at the centre of each plate for uniform outgrowth of mycelia under aseptic conditions inside an LAF (Fig. 4). At the base of few Petri plates containing used up culture for the study, two perpendicular lines were drawn to obtain the centre of the medium which was used up as a guide for positioning of the inoculum. The plates were then resealed and incubated at 26-27°C in an incubator. Continuous monitoring for growth and contamination has been done on a regular basis. This helped to observe the growth of mycelia in PDA media.

Test tubes: Sterilized substrate test tubes were inoculated with three culture plugs (9.5 mm) by the help of inoculation loop to introduce the plugs into each test tubes. The substrate tubes were incubated at room temperature (27-30°C) inside the LAF in the University Pharmacy laboratory. Daily monitoring was done to check the growth and contamination. This assisted in detecting the growth progress in sawdust substrate.

Measurement of colony diameter observed: For the Petri plates, the mycelium yield of *P. pulmonarius* was determined by measuring the diameter of the colonization using a ruler across the Petri plate horizontally in centimetres. The outer circumference of the colony was marked around from three angles with the mean of the 3 measurements recorded as the final value.

For substrate test tubes, the length of mycelial growth was measured across the test tube vertically from the top of substrate level till the end tip of mycelial growth using a ruler in cm. The length was measured from 3 angles or sides and means of the figures were recorded as the final value. The number of days for the growth measurement was recorded for each alternative days.

The measurement of the triple replicates was summed up to calculate the mean value. The growth rate was assessed by using the formula given below.

$$Growth\ rate = \frac{Colony\ diameter\ on\ the\ last\ day}{No.of\ days\ measurement\ was\ taken\ after\ inoculation} \quad [11]$$

Analysis: The obtained results of the growth were measured in centimetres and recorded for three replications. The data collected in this study were statistically analysed with one-way ANOVA and mean result with ANOVA using Statistical Package for Social Science (SPSS) with visible significance in the growth of media, salinity and strain.

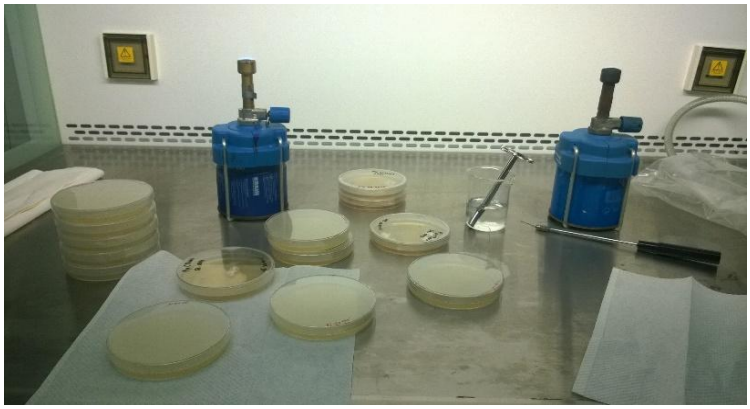

Fig. 4. PDA culturing arrangement in the LAF

3. RESULTS AND DISCUSSION

3.1 Study on the Growth of *P. pulmonarius*

Both the strains of the oyster mushroom exhibited desirable growth in both PDA and substrate medium as tabulated below in Table 1. Full colonization was observed for PDA plates with 7- 8 days while substrate tubes displayed full growth within 14-16 days of inoculation. Clear distinct growth rates were observed for different salinities with different water samples and with a little dissimilarity seen in the growth of two strains. The statistical analysis of variance of colony diameter recorded per day for different farm water, different salinity, growth medium and mushroom strain shown in Table 2.

3.2 Effect of Chemical Parameters

3.2.1 Salinity relation to growth rate observed

The productivity of the mycelial growth was successfully observed only in low concentrations while leaving the higher concentrations uncolonized (Fig. 5). To be specific, best growth with full colonization of the plates and test tubes were observed in 0% and 1% NaCl for both China and Thailand strains, while 5% and 10% NaCl showed zero growth even after they were observed until the very end of the experiment with an exception to W2 (Fig. 6). The increase in salinity concentration increased the number of days for complete colonization. Hence the fastest and highest mycelial extension was seen in 0% followed closely by 1%.

From this observation, it can be assumed that salinity affects the growth and expansion of mycelia of *P. pulmonarius* at various levels of concentrations for the NaCl salt tested. This corresponds with other studies conducted so far, confirming that vegetative growth and development of mushrooms are inhibited with reduction or retardation in growth under salt stress as mentioned by Ayodele and Ojoghoro [12]. The highest mycelium growth rate observed was in PDA media (0.41 cm/day) and this medium has been proved to be highly supportive of the mycelial growth of mushrooms by authors such as Jeffers and Martin [13]. This might be illustrated due to the osmotic or ionic effect or combination of both, that interferes with the uptake of water by salts which are highly essential for yielding of mushroom.

Table 1. Average growth rate observed for both the strains in PDA and sawdust substrate medium

		Average daily mycelial growth of colony diameter of 3 replicates (cm)								Average growth observed per day
		1	2	3	4	5	6	7	8	
China	PDA	0.6	1.1	1.9	2.7	3.4	4.1	4.4	4.50	0.56
Strain	Sawdust Substrate	0.5	.9	1.5	2.3	2.9	3.5	4.6	5.24	0.37
Thailand	PDA	0.7	1.0	1.6	2.0	2.5	2.9	3.2	3.52	0.44
strain	Sawdust Substrate	0.2	0.6	0.8	1.0	1.1	1.2	1.3	1.45	0.10

Table 2. Analysis of variance showing mean results with standard deviation for different salinity, growth medium, water samples and strains on colony diameter observed per day

Growth observed per day	Colony diameter recorded per day			
	* different salt concentrations	* water contents collected	* Type of medium used for growth	* Mushroom strains
1	110.08**	00.46^{ns}	11.55**	00.25^{ns}
2	162.22**	00.37^{ns}	05.55*	02.08^{ns}
3	144.54**	00.22^{ns}	06.75*	04.88*
4	146.00**	00.26^{ns}	04.54*	09.45*
5	150.25**	00.13^{ns}	05.54*	11.31*
6	145.68**	00.08^{ns}	06.13*	13.38**
7	145.49**	00.10^{ns}	02.51^{ns}	17.13**
8	148.93**	00.13^{ns}	01.23^{ns}	17.26**
Average growth observed per day	116.66**	00.05^{ns}	18.40**	09.97*

***- absolute significance, *- significant and ns- not significant*

Table 3. Report of means for growth analysis done with different salinity concentrations

Different salt concentrations used	Mean of colony diameter recorded per day (cm) ± standard deviation								Average growth observed per day
	1	2	3	4	5	6	7	8	
0% NaCl (Control)	1.15 ± 0.61	1.94 ± 0.77	3.05 ± 1.30	4.35 ± 2.00	5.29 ± 2.43	6.13 ± 2.81	6.93 ± 3.10	7.50 ± 3.15	0.77 ± 0.379
1% NaCl	0.81 ± 0.42	1.59 ± 0.72	2.44 ± 1.17	3.37 ± 1.42	4.37 ± 1.79	5.24 ± 2.29	6.15 ± 2.65	6.77 ± 2.97	0.68 ± 0.35
5% NaCl	0.04 ± 0.12	0.10 ± 0.27	0.15 ± 0.41	0.22 ± 0.62	0.24 ± 0.70	0.26 ± 0.76	0.37 ± 1.13	0.44 ± 1.37	0.03 ± 0.09
10% NaCl	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

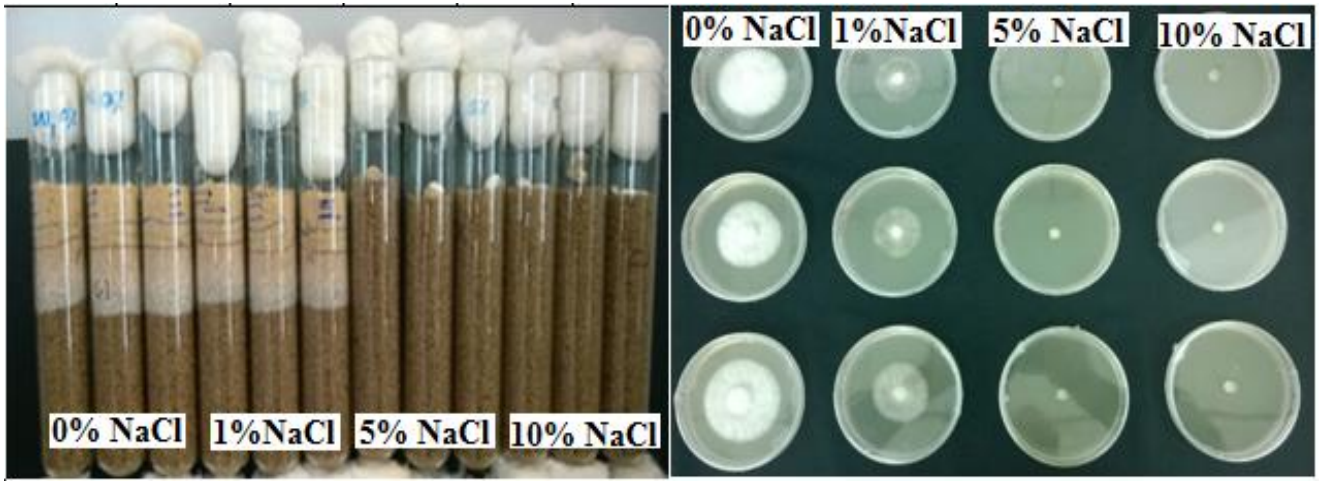

Fig. 5. Growth of mycelia observed in PDA and sawdust substrate

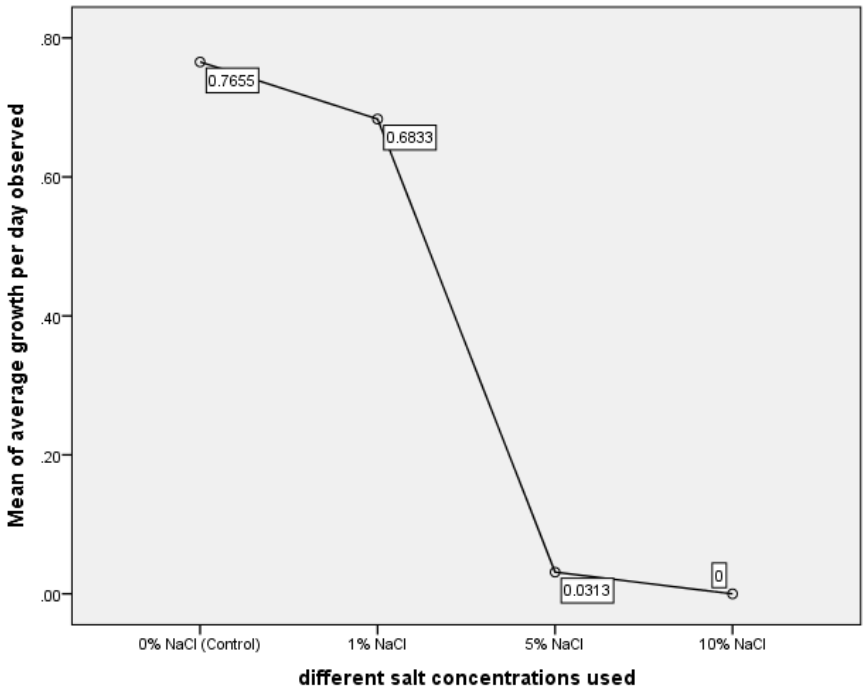

Fig. 6. Growth of Mycelia observed for different Salinities

The analysis of variance for mycelia growth given in Table 2 shows a significant growth per day (116.66**). The results with more elaboration of values are shown in Table 3 which gives a report of the ANOVA table in details with the means of the growth rates recorded and standard deviations.

3.2.2 Disparity in mycelial growth rate for water samples

Vegetative growth of both the strains of *P. pulmonarius* showed differed growth rates in both Petri plates and test tubes for each water sample with standard deviation ≤ 1 within the groups as seen in Table 6. The difference in growth if placed into an order of growth, it shows the results as following Tables 4 and 5.

For PDA plates:

Table 4. Growth flow in the increasing order of mycelia W1- Lab Water, W2- Nas-Agro Farm Water, W3- GanoFarm Water & W4- 7* agricultural farm water

China	W1 → W3 → W2 → W4
Thailand	W2 → W3 → W1 → W4

For Sawdust substrate test tubes:

Table 5. Growth flow in the increasing order of mycelia W1- Lab Water, W2- Nas- Agro Farm Water, W3- GanoFarm Water & W4- 7* agricultural farm water

China	W3 → W4 → W2 → W1
Thailand	W2 → W3 → W1 → W4

Best overall mycelial yield performance was observed in a water sample collected from Nas-Agro farm (W2) illustrated in Fig. 7. W2 in saw dust substrate medium gave better results in showing expansion of vegetation in the substrate for three salinity (0%,1%, and 5%) while other samples failed to give the same results but only for 2 salinities (0% and 1%). W2 also showed second best growth performance for Thailand strain in PDA plates. Growth curves for both the strains are shown in Figs. 8 - 11 for 0% and 1% salinity in four water samples with colony diameter per day as y-axis and number of days growth was observed in the x-axis. This data subjected to analysis of variance showed no significance (0.05^{ns}) in growth rate within the water samples collected (Table 2).

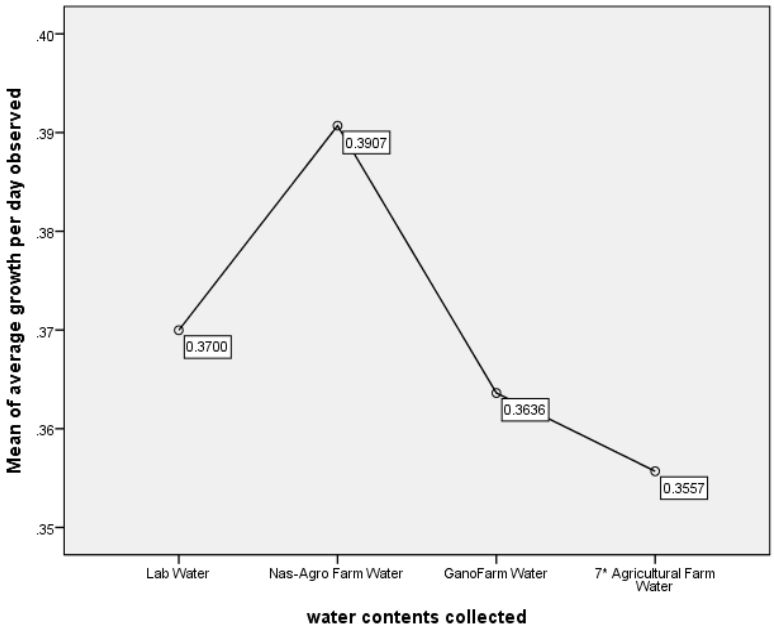

Fig. 7. Average growth rate of mycelia observed for each water sample collected

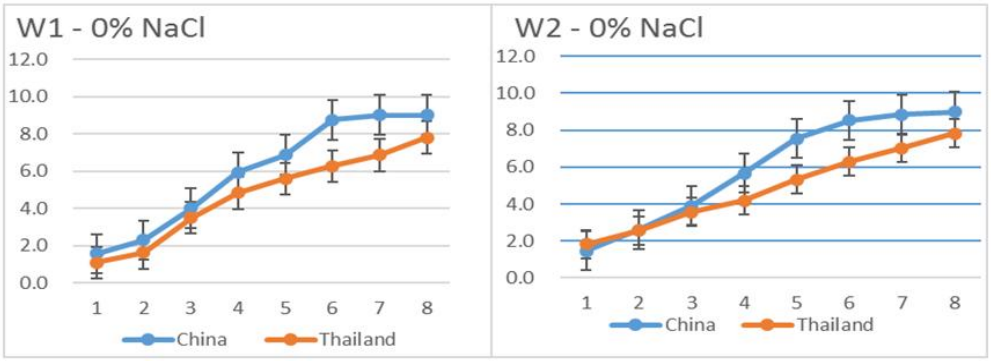

Fig. 8. Growth curve observed for both the strains at 0% NaCl in W1- Lab Water and W2- Nas-Agro farm water

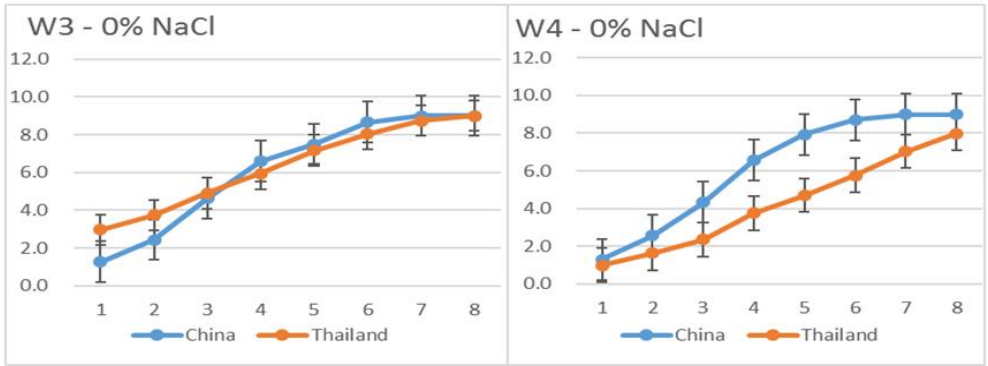

Fig. 9. Growth curve observed for both the strains at 0% NaCl in W3- Gano-farm Water and W4- 7* Agricultural farm water

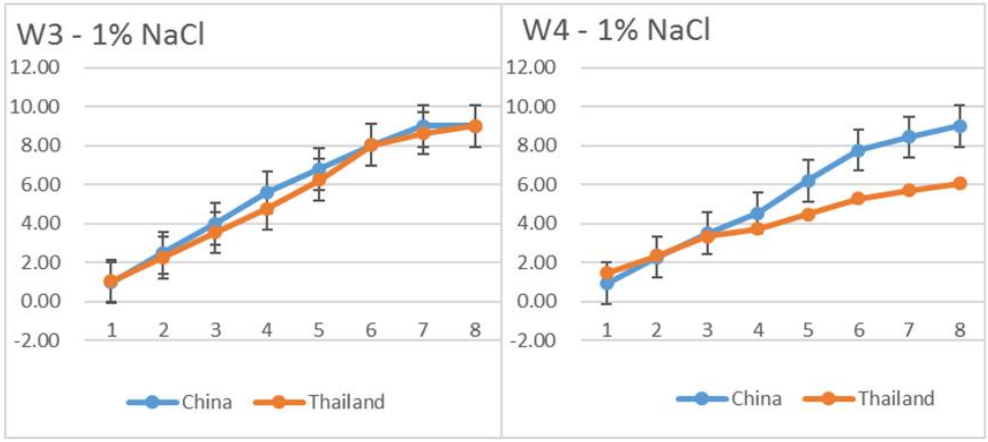

Fig. 10. Average growth rate of mycelia observed for each water sample collected

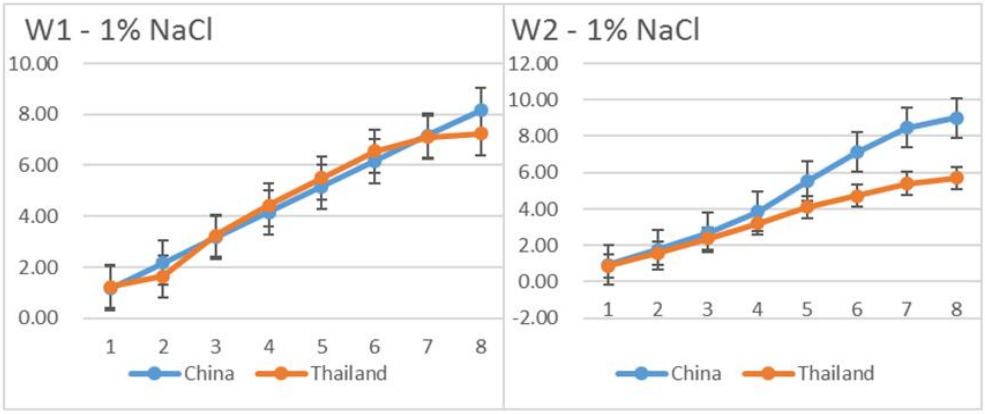

Fig. 11. Growth curve observed for both the strains at 1% NaCl in W1- Lab water and W2- Nas-Agro farm water

The significance of ANOVA results for growth observed within groups of a water sample collected in Table 2 which is elaborated in the following Table 6 giving a more clear view of data with standard deviation. The data shows that W2 showed better results followed by W1 then W3 and last W4 which can be read from the average growth rate recorded. The growth means differing in the data implies that growth did vary between the water samples collected.

3.2.3 Effect of physical parameters on the growth

The medium of growth taken as a physical parameter did show a distinctive significance (18.48**) with the recorded data subjected to ANOVA as observed in the Fig. 12. In PDA full colonization of fungi was observed within a short number of days at a fast pace in contrast with the sawdust substrate where the initial development was at a low pace and full colonisation took half more additional time. By looking at the following Figs. 4, 8 and Table 7 the difference in mushroom growth can be observed much clearly. Rapid growth rate and high-frequency production were observed in PDA plates while in sawdust substrate test tubes. Growth rate noted was initially gradual but in later stages, it showed a high rate of growth with better performance.

This result goes in hand with the study done on different media and other factors for the growth of oyster mushroom, the highest mycelial growth rate (0.24 cm/day) observed in the potato dextrose agar (PDA) media with lowest mycelial growth found in YEA media (0.11 cm/day) and the density of mycelium on PDA higher than other culture media [14].

Table 6. Report of means for growth analysis done with water samples collected

Water contents collected	Mean of colony diameter recorded per day (cm) ± standard deviation								Average growth per day
	1	2	3	4	5	6	7	8	
Lab Water (W1)	0.49 ± 0.60	0.82 ± 0.90	1.35 ± 1.58	1.80 ± 2.18	2.31 ± 2.74	2.76 ± 3.33	3.28 ± 3.94	3.63 ± 4.32	0.37 ± 0.46
Nas-Agro Farm Water (W2)	0.50 ± 0.53	0.90 ± 0.87	1.36 ±1.35	1.98 ±1.10	2.54 ±2.62	3.00 ±3.13	3.60 ±3.67	3.99 ±4.08	0.39 ± 0.43
GanoFarm Water (W3)	0.59 ± 0.81	1.03 ±1.28	1.57 ±1.97	2.21 ±2.69	2.65 ±3.17	3.04 ±3.64	3.39 ±4.02	3.61 ±4.18	0.37 ± 0.45
Agricultural Farm Water (W4)	0.44 ± 0.51	0.88 ±1.01	1.36 ±1.58	1.95 ±2.29	2.40 ±2.90	2.83 ±3.40	3.19 ±3.80	3.48 ±4.13	0.36 ± 0.45

Table 7. Report of means for growth analysis done with medium of growth

Type of medium used for growth	Mean of colony diameter recorded per day (cm) ± standard deviation								average growth per day
	1	2	3	4	5	6	7	8	
PDA	0.65 ± 0.75	1.08 ± 1.17	1.71 ± 1.84	2.33 ± 2.52	2.95 ± 3.14	3.50 ± 3.70	3.80 ± 3.98	4.01 ± 4.17	0.50 ± 0.52
Sawdust Substrate	0.35 ± 0.41	0.74 ± 0.83	1.10 ± 1.32	1.63 ± 1.98	2.00 ± 2.43	2.3 ± 2.87	2.93 ± 3.64	3.35 ± 4.12	0.24 ± 0.30

Table 8. Report of means for growth analysis done with mushroom strain

Mushroom strains used	Mean of colony diameter recorded per day (cm) ± standard deviation								Average growth observed per day
	1	2	3	4	5	6	7	8	
China Strain	0.52 ± 0.55	1.01 ± 1.02	1.67 ± 1.70	2.48 ± 2.53	3.14 ± 3.16	3.77 ± 3.77	4.46 ± 4.33	4.87 ± 4.69	0.47 ± 0.48
Thailand strain	0.48 ± 0.69	0.80 ± 1.01	1.15 ± 1.50	1.48 ± 1.90	1.80 ± 2.30	2.05 ± 2.64	2.26 ± 2.88	2.48 ± 3.11	0.27 ± 0.38

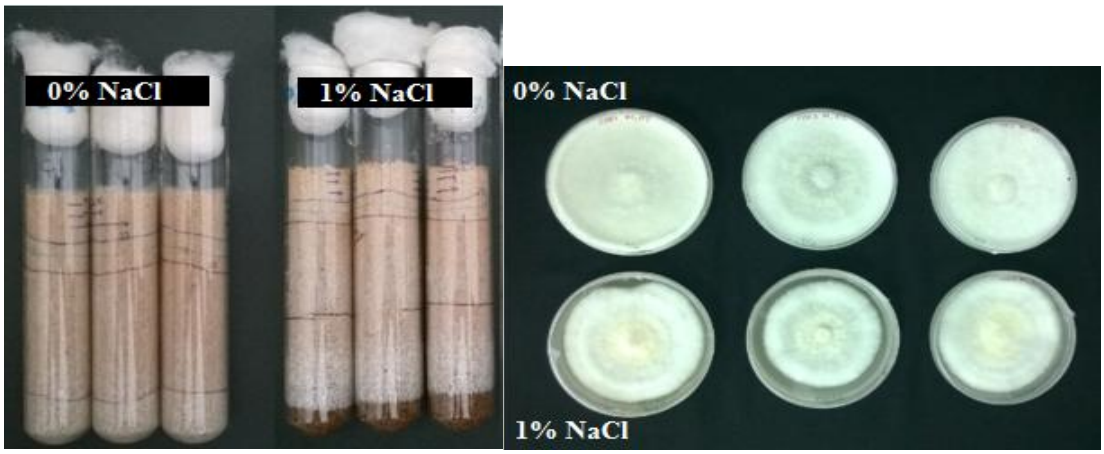

Fig. 12. Colonization observed on the last day of observation

The difference in mycelial growth rate in both the mediums can be read from the Table 7 as 0.5 cm in PDA and 0.24 cm in sawdust substrate for average growth recorded per day.

3.2.4 Dissimilarity in mycelial growth of the two strains

The strains of Thailand and China showed noticeable fluctuations in their growth rate and density in both the media under salt stress and different water with a significance of 9.97** for analysis of variance. The data given in the Table 8 gives a significant difference between both the strains for average growth observed per day in their mycelial growth with standard deviations. China strain exhibited fast development (0.47 cm) with a denser colony and uniform growth in all the plates while Thailand strain trailed behind at a moderate growth rate (0.27 cm) with a less dense colony and uneven growth in the vast majority of the development.

From the average growth rate per day given in Table 8 above, China strain shows much higher growth while Thailand strain shows lower growth rate with a difference of values adding to the observations made. This suggests that variation in the growth performance does occur although the two strains belong to the same species as mentioned in a previous study [15,16] on different strains which also demonstrated different performances on the ability to colonise the growth medium [17].

4. CONCLUSION

The utilization of different materials and other possible resources for the creation of active mycelia is a significant improvement as it could give another approach to waste administration and enhancement of mushroom growth in the agricultural field. In the current study, the positive effects of appropriate strain, farm water and salinity level on the mushroom mycelium growth were shown. Moreover, combination or supplementation treatment might be embraced to enhance the outcome acquired on PDA and sawdust substrate. Hence, further research in exploring the growth of this mushroom in distinctive physicochemical parameters such as pH, growth medium, salinity and water for advancement in the culturing is suggested.

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COMPETING INTERESTS

Authors have declared that there is no competing interests exist regarding the present work is concerned.

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