BIBLIOGRAPHY

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ABSTRACT

A study determining culture medium appropriate for spore germination, comparing spore punch and agar block method to obtain single spore, determining effect of length of time of exposure of germinated spore to ultra violet light on dikaryotization and determining for incidence of dikaryotic mycelium production was conducted at the Plant Pathology Laboratory, Benguet State University from October 2007 to October 2008.

Results revealed that malt extract agar is the most suitable culture medium for spore germination and growth after two days of incubation as compared to potato dextrose agar which yielded shorter mycelial length at the same period of incubation and water agar which yielded a germinated spore after six to ten days of incubation.

For obtaining a well isolated single spore, spore punch method proved to be more reliable than that of the agar block method. Spore punch method gives higher chance of obtaining a single spore and is less laborious than that of the agar block method.

Monokaryons exposed to ultra violet light at different lengths of time from 10 to 60 minutes did not produce a dikaryon. Rather, an inhibition zone was very evident in all treated plates except that exposed to UV light for 60 minutes. This was subjected to

mycelial diameter measurement. Mycelium from both parents and the supposed dikaryon was measured and compared but no difference was observed.

It is recommended that studies be conducted testing other mutagenic agents using the given protocols on culture media used and technique on single sporing.

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INTRODUCTION

Background of the Study

Mushrooms are fleshy spore-bearing fruiting bodies of fungi. Most are saprophytic, wood-decomposing fungi. Thus, many are seen growing on twigs, chips of wood, stump of logs and soil. Kaul (1997) mentioned that mushrooms appear almost everywhere – deserts, open fields, gardens, farmyards, marshes, forests, alpine-areas in almost all the biomes on this planet. However, the majority are confined to wooded areas. From the earliest days, mycologists recognized the fact that both large and small fungi are ecologically connected to herbaceous plants and tress amongst which they grow.

Because of their medicinal properties, nutritional content and potential for the global market, mushroom cultivation is growing and gaining popularity. A lot have cited of their effect on the immune system. *Ganoderma*, for example is considered a natural regulator of the immune system. Oyster mushrooms are best known medically for their cardiovascular and cholesterol-controlling benefits. *Shiitake* on the other hand is recognized for its antitumor action. Mushrooms are also good sources of protein, carbohydrates and fiber.

The demand for good quality of mushrooms is high especially in the urban areas due to the emergence of mushroom producers that compete for market. Mushroom standards are then raised to that which can produce more and that with better quality than of the existing criteria.

It should be noted that degeneration occurs after frequent subculturing from the original strain. Cultivars used for commercial spawn production that were maintained on various agars or cereal grains with periodic subculturing of growing mycelium to a fresh

medium were reported cases of culture degeneration periodically (http://pubs.cas.psu.edu). It is also commonly observed that tissue cultures often give lower yields than the original cultures (www.krishiworld.com). Tissue culture is in a sense cloning. It gives rise to mushrooms with similar characteristics with that of the original, thus, no room for strain improvement. Improving mushroom strain is necessary to likewise improve their yield and quality, enabling them to compete in the market. Hybridization calls an important role in this intent. According to Eger (1978) as cited by Arias *et al.* (2000), large number of hybrids, obtained by pairing monosporic cultures need to be cultivated to evaluate their production characteristics. This would bring about a trial and error process during the selection of promising hybrids which would take quite sometime.

Importance of the Study

Breeding is the only controllable means by which desired traits of different stocks or strains can be combined. The quality of the crop depends as much upon the inherited potential of the strain used as does upon conditions for growth and development (Raper, 1978). Fritsche (1978) said that by continued selection of mushrooms, bad characteristics are eliminated.

Hybridization offers greater possibility for genetic improvement because of the exchange of genetic information. This results from the fusion of two haploid nuclei.

Regardless of this, most spawn is made with mycelium from a stored culture, rather than mycelium whose parent was a spore. This is because spores are likely to yield a new strain and performance would be unpredictable (http://pubs.cas.psu.edu). The issue of unpredictability might be reduced by testing each hybrid. Arias *et al*. (2000) obtained

high yielding *Pleurotus ostreatus* by cultivating hybrids of this species resistant to 2 Deoxyglucose (2DG).

This study aimed to:

1. determine culture medium appropriate for spore germination;
2. compare techniques for single sporing;
3. determine the effect of length of time of exposure of germinated single spore to Ultra Violet (UV) light on dikaryotization; and
4. determine for incidence of dikaryotic mycelium production.

Time and Place of the Study

This study was conducted at the Plant Pathology Service Laboratory from October 2007 to October 2008.

REVIEW OF LITERATURE

The Mushrooms

Mushrooms are good cash crops; they are rather easy to grow and are brimming with protein, B vitamins and minerals. They also have medicinal properties like anti-cancer (Oei, 2005).

Because of their medicinal properties, nutritional content and potential for the global market, mushroom cultivation is growing and gaining popularity. A lot have cited of their effect on the immune system. *Ganoderma*, for example is considered a natural regulator of the immune system. Oyster mushrooms are best known medically for their cardiovascular and cholesterol-controlling benefits. *Shiitake* on the other hand is recognized for its antitumor action. Mushrooms are also good sources of protein, carbohydrates and fiber.

Importance of Hybrid Mushrooms

The purpose of breeding is to combine desired traits present in separate individuals using controlled crosses and selection of offspring (Sonnenberg, 2007).

Several studies showed the significance of breeding mushrooms. A study conducted by Arias, *et al.* (2000) resulted to six hybrids that showed improved fruiting characteristics and had maximum productivity. Anderson, *et al.* (2001) found out that hybrid off-white strains of *Agaricus bisporus* exhibited intermediate susceptibility to *Trichoderma harzianum*, with mean yield losses of 56% to 73%. According to Miller (2005), hybridization of *Cordyceps* amplified target medicinal compounds to five times its potency. Hybridization concentrates the dosage, lowering volume ingested

substantially over the wild varieties. With hybrid cordyceps, we obtain cordyceps for target medicinal compounds, vigorous or fast growing, and resistance to infection with other organisms. Hybridization also may modify the substrate used in cultivating some mushrooms. Bak *et al.* (2005) states that FRI 169, a hybrid shiitake mushroom turned out to be a superior strain for sawdust-based cultivation.

Hybridization

Unlike plant and animal breeding, mushroom breeding is a relatively new applied science. This is not surprising since only recently have mushrooms been produced commercially on a large scale (Sonnenberg, 2007).

Breeding is the only controllable means by which desired genetic traits of different stocks or strains can be combined. In the higher fungi, its minimal requirements are: a recognizable sexual interaction between mated strains, fruiting competence, meiosis and viability of spores (Raper, 1978). She further says that the quality of the crop depends as much upon the inherited potential of the strain used as does upon conditions for growth and development. By continued selection, bad characteristics are eliminated (Fritsche, 1978).

The true success of the hybrid would be if it produces fruits that has spores. Only at this stage would the true hybrid strain be accomplished. No genetic information is exchanged between two strains until their separate haploid nuclei have fused and then undergone meiosis. Recombination would then occur between the two separate strains forming a third strain. Its offspring (spores) would be new combinations of the two donor strains. Simply cloning the original mating that fruited will be a hybrid as well, but not a true hybrid, because there has been no recombination between the two strains, no mixing

of genes. There has simply been a successful coexistence of two haploid nuclei, one from each strain, acting independently, but together to create fruits (www.shroomery.org). Thus, the importance of single sporing.

Elliott and Langton (1981) as cited my Yadav *et al.* (1999) emphasized that the hybridization methods based on the combination of non-fertile, homokaryotic single spore cultures offer better prospects for genetic improvement than the traditional selection methods. Yadav made use of single sporing technique by serially diluting an obtained spore mass and isolating the germinated single spore cultures from the serial dilution. Similarly, Arias *et al.* (2000) produced dikaryons by pairing monokaryotic strains, either neohaplons or single spore isolates. Sexual compatibility was assessed by the positive clamp connection seen under the microscope.

In the process, not all sub-strains within a strain will fuse, some are completely incompatible. There is a zone of zero growth between the two strains on the Petri plate (www.shroomery.org); (www.nwbotanicals.org). Holliday (2004) reported that when different strains were inoculated together onto one Petri dish, the strains grew towards each other until they formed a zone of inhibition. But through the addition of snake venom to the agar, the zone of inhibition becomes short lived until the mycelial strands fuse and exchange nuclear material through their venom-weakened cell wall. Miller (2005) also observed that snake venom changes the form of the *Cordyceps* by breaking down its cell walls, rather than being added into the final product and indicated that snake venom is an essential catalyst for hybridization allowing the fusion of the two differing Deoxyribonucleic acids (DNA's).

Ultra Violet (UV) is a strong physical mutagen and it is directly absorbed by the DNA bases (Fincham *et al.*, 1975). Perera *et al.* (2005) observed the conversion of ergosterol in mushrooms to vitamin D_2 when exoposed to UV.

Santos *et al.* (1989) were able to obtain thermotolerant and high alcohol yielding mutant of *Saccharomyces cerevisae* when treated with acridine mustard (AM) and nitrous acid (NA). Accordingly, acradine mustard is a frameshift mutagen and nitrous acid is a demeaning agent. Santiago *et al*, (1991) claimed that nitrous acid is an effective mutagen for the isolation of mutant in A*garicus bisporus* and *Lentinula edodes*. He added that the nitous acid dose depends upon the type of species and nature of the desired mutants.

With the occurrence of mutation, performance of the new strain is unpredictable. Hybrids must be cultivated to evaluate their production characteristics. Arias *et al.* (2000) reported that the use 2-deoxyglucose was used to select high yielding resistant hybrids.

2-Deoxy-D-glucose has a molecular formula of $C_6H_{12}O_5$. It is a molecule which has the 2-hydroxyl group replaced by hydrogen, so that it cannot undergo further glycolysis (http://en.wikipedia.org). 2-Deoxyglucose is a rare and natural monosaccharide. It is the basic structure of the anticancer drugs such as daunomycin, adriamycin, carminomycins and antibiotics.

2-DG is a white crystalline hydroscopic powder. It is odorless, tastes sweet and very soluble in water, partially soluble in hot methanol, ethanol, acetone, and butanol. It is not soluble in ether, chloroform, petroleum ether and toluene. 2-DG is a polyhydroxy aldehyde with reducing ability can react with Fehling or Benedict reagent and produce red or yellow precipitation of copper oxide. 2-DG acts to inhibit the phosphorylation of a glucose molecule that produces glucose-6-phosphate in the glycolysis cycle, therefore

inhibiting the production of ATP and can also decrease the temperature in muscle cells. It can restrain viral infections and fermentation, microbes and cancer cell growth. It also controls metabolism and possesses physiological and therapeutically effects. (http://www.2dgpro.com)

The effect of several concentrations of the toxic analogue of glucose, 2-deoxy-D-glucose (2-DG), was observed on the rate of apical growth of strains of *Pleurotus ostreatus* using different carbon sources. The growth of the strains under these conditions distinguished between tolerant and sensitive strains. It was observed that the phenotype which was tolerant to 2-DG was correlated with high productivity of the strains in pilot production farms (Sanchez *et al.,* 1996).

To eliminate the trial and error process during hybridization, Arias *et al.* (2000) used 2DG in the selection of recovered *Pleurotus ostreatus.* Resistance to this toxic analogue of glucose had been used to select overproducing microorganisms. In their study, six hybrids showed resistance to 2-DG out of the original thirteen hybrids. The 2-DG resistant hybrids showed required 62 days to fruit and achieved maximum productivity in a fruiting period of 28 days, with fruit bodies of 18.5g average weight.

There were similar results of that of Kirimura *et al*. (1992) with *Aspergillus niger* mutants resistant to 2-DG that produced citric acid earlier than sensible individuals and that of Sanchez and Viniegra – Gonzales (1996) with *Pleurotus ostreatus* resistant strains gave higher yield as reported by Arias *et al.* (2000)

MATERIALS AND METHODS

Collection of Specimens

Ten mushrooms were collected, five each from both white (*Pleurotus cornucopiae*) and gray (*Pleurotus ostreatus*) oyster species. These were characterized as to 1.) size, shape, and color of the cap; 2.) size, shape, color of spores and; 3.) form of hymenophore.

Collection of Spores

Collection of spores was done by removing the cap and laying it faced down. Half of the cap was laid over white paper and the other half on black paper. After hours, the deposited spores were collected for single sporing and for microscopic characterization.

Calibrating the Microscope

Calibration of the microscope was done by inserting the ocular micrometer into the eyepiece and the stage micrometer on the stage. Using the low high power objective (40x), zero (0) point of both stage and ocular micrometers sere set to coincide with each other. The ocular divisions that cover the space between zero and the coincident line were counted. Calibration Factor (CF) or Calibration Constant was calculated using this formula:

$$CF = \frac{\text{n divisions of stage micrometer}}{\text{n divisions of ocular micrometer}} \times 10 \text{ units/division}$$

Protocols for Optimizing Spore Germination

Effect of the Kind of Media and Species Used

Spore germination of each mushroom species was tested on differential media namely. Each treatment was replicated four times and arranged in a factorial completely randomized design (CRD) with mushroom species as factor A and different media used as factor B. The treatments were as follows:

Factor A

A_1 – *P. ostreatus*

A_2 – *P. cornucopiae*

Factor B

B_1 – Water agar

B_2 – Potato Dextrose Agar (PDA)

B_3 – Malt Extract Agar (MEA)

Commercial sterilized distilled water was poured into the Petri dish containing the spores after which 0.1 mm was obtained and spread over the differential media. Plates were incubated at 23°C and presence of germinated spores was observed after 48 hours. Treatments were replicated four times.

Single Sporing Technique

Two modified techniques of single sporing according to Manzanares *et al.* (1994) and Webster *et al.* (1981) were tested. Treatments used were:

S_1 Agar block method

S_2 Spore Punch Method

For the agar block method, the media was cut into cubes at approximately 1 mm x 1 mm then observed under the microscope for well isolated single spores.

The second method is the use of spore punch. An agar disc bearing a germinated spore was obtained using a spore punch mounted in the microscope.

Agar blocks and spore punches containing a single spore were transferred to the media that gave the best result on initial test of media. Each plate contained a spore each of white and gray oyster mushroom. Treatments were replicated four times.

Hybridization between species was also tested by inoculating two spores from the same kind of mushroom (i.e. *P. ostreatus* with *P. ostreatus* and *P. cornucopiae* with *P. cornucopiae*) but from different fruiting bodies into a Petri dish.

Isolates that produced mycelia were cut and transferred to plates and were paired with other isolates that also produced mycelia.

Protocols for Optimizing Hybridization

Exposure Time to Ultra Violet (UV) Light

Petri plates containing the single spores were exposed to Ultra Violet (UV) light with a distance from the lamp of 15 cm (Santiago, Jr., *et al.*). The treatments were:

T_0 – Control

T_1 – 10 minutes

T_2 – 20 minutes

T_3 – 30 minutes

T_4 – 40 minutes

T_5 – 50 minutes

T_6 – 60 minutes

Presence of Dikaryotic Mycelia

Treated isolates were observed under the microscope for the presence of clamp connection. Additionally, mycelia were taken from both parent spores and from meeting points of both mycelia which is treated as a possible hybrid candidate and were measured as to mycelial diameter.

Data Gathered

The data gathered were the following:

1. Physical description of parent mushroom. Mushroom fruiting bodies were described as to (a) size of cap, (b) shape of cap, (c) color of cap and (d) form of hymenophore.

2. Microscopic description. The size, shape and color of spores were described.

3. Effect of media on germination. Presence of germination was noted as well as length of mycelium.

4. Presence of clamp connection.

RESULTS AND DISCUSSIONS

Physical Description

Mushroom characterization is presented in Table 1. The mushrooms used were harvested after 36 hours from pinhead formation. Results reveal that white oyster mushroom (*Pleurotus cornucopiae*) is bigger in size than gray oyster mushroom (*Pleurotus ostreatus*). Both have a convex, fan-shaped cap and are gilled.

Table 1. Physical description of parent mushroom

Specimen	Size of cap (cm)	Shape of cap	Color of cap	Form of hymenophore
P. cornucopiae	5.2	Convex, fan-shaped	White	Gilled
P. cornucopiae	5.0	Convex, fan-shaped	White	Gilled
P. cornucopiae	5.4	Convex, fan-shaped	White	Gilled
P. cornucopiae	5.2	Convex, fan-shaped	White	Gilled
P. cornucopiae	5.0	Convex, fan-shaped	White	Gilled
P. ostreatus	5.0	Convex, fan-shaped	Gray	Gilled
P. ostreatus	4.8	Convex, fan-shaped	Gray	Gilled
P. ostreatus	4.0	Convex, fan-shaped	Gray	Gilled
P. ostreatus	4.7	Convex, fan-shaped	Gray	Gilled
P. ostreatus	4.8	Convex, fan-shaped	Gray	Gilled

Microscopic description

Spore shape for both mushrooms is kidney-shaped with white to pinkish spore print. At 500x magnification, spore size for both mushrooms measures 10.71 x 3.57.

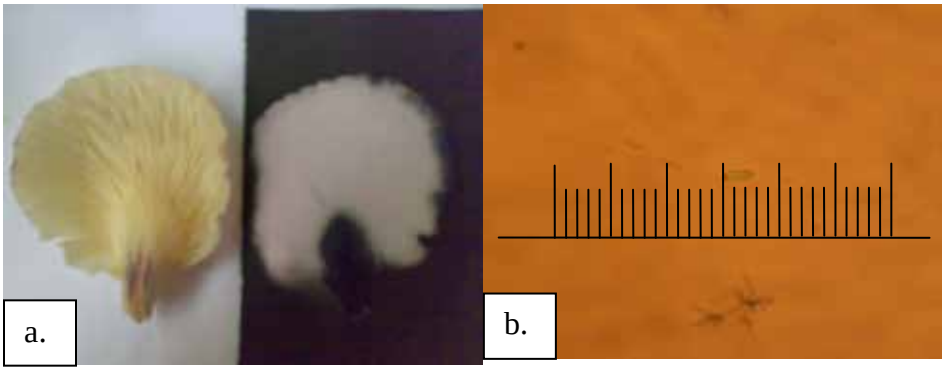

Figure 1. (a) White to pinkish spore print and (b) a spore of *P. cornucopiae.*

Effect of the Kind of Media and Species Used

Spore germination was observed after two days from inoculation. Table 2 shows that there are no significant differences on the length of the mushrooms as affected by the species. These signifies that the *P. cornucopiae* and white *P. ostreatus* are not significantly different from each other in terms of there mycelium length. Moreover, the effect of the media on mycelia length is highly significant. Table 2 shows that MEA gave significantly higher mean length of 47.77µ. followed by PDA and water agar with mean mycelia length of 29.018 µ and 0.000 µ, respectively.

Analysis further shows that there are no significant effects of species and medium interaction on the length of the mycelium. This means that the species and the medium are independent from each other. That the differences on the length of the mycelium is not a result of the interaction of the species and the medium, rather, it is either a result of the effect of the species or of the medium alone. In this case, the significant effect ion the kind of media used.

Table 2. Mean mycelium length as affected by species and media used

	Mean Length (µ)
Species	
P. ostreatus	27.83[a]
P. cornucopiae	23.36[a]
Medium	
MEA	47.77[a]
PDA	29.018[b]
Water Agar	0.000[c]
Species x Medium	ns
CV (%)	26.34

Means of the same letter are not significantly different from at each other at 5% level of significance by DMRT.

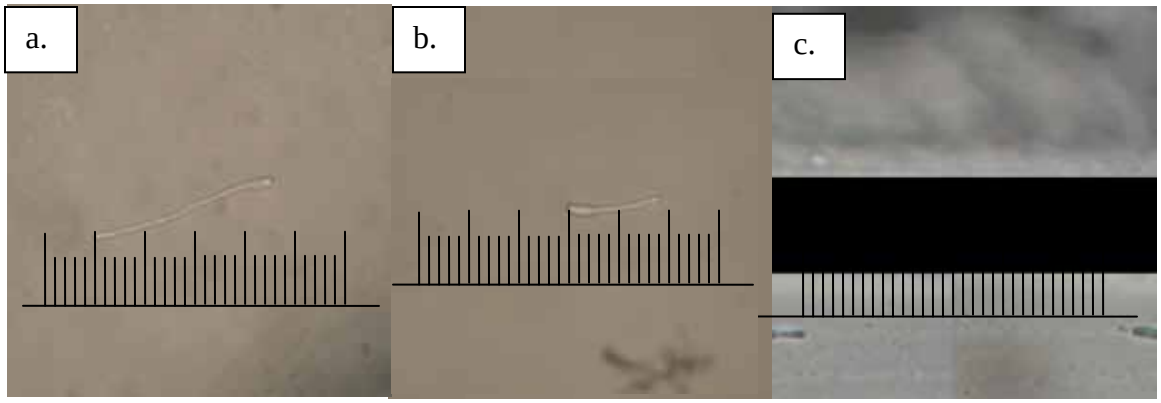

Figure 2. Geminated spores of *Pleurotus ostreatus* on (a.) MEA, (b.) PDA 2 days after inoculation and (c.) Water Agar 10 days after inoculation at 500x.

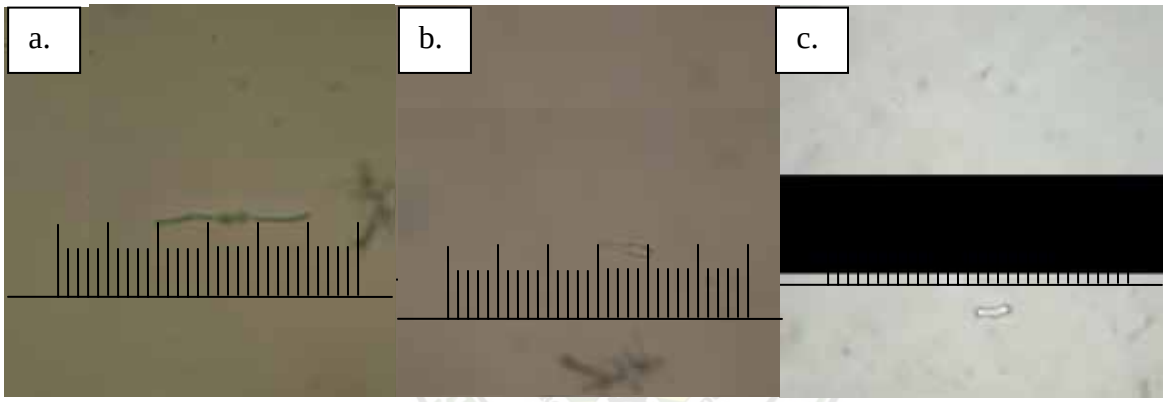

Figure 3. Germinated spores of *P. cornucopiae* on (a) MEA, (b) PDA 2 days after inoculation and (c) Water Agar 10 days after inoculation at 500x.

Appropriate Technique of Single Sporing

Single sporing methods were tested on MEA as it is a promising medium for spore germination and growth. Spore punch method generated higher possibility of obtaining a well isolated, germinated, single spore as compared to the agar block method. Moreover, spore punch method is a more rapid way to obtain a single spore than that of the agar block method where a lot of agar blocks should be made to increase the chance of obtaining a single spore.

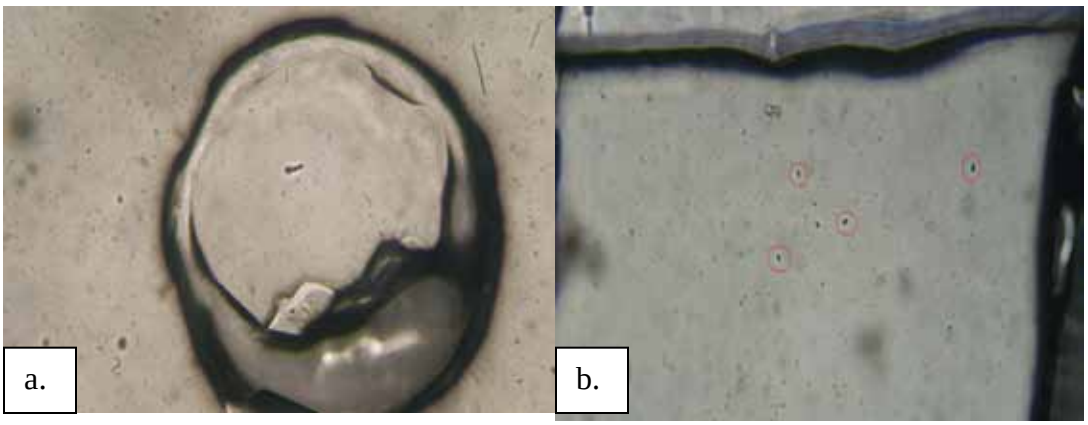

Figure 4. Comparison of single spore methods (a) spore punch and (b) agar block method at 100x for *P. cornucopiae.*

Mycelia emerging from the single spore was observed after 6 to 10 days from incubation at 23ºC only from spores of *Pleurotus ostreatus.*

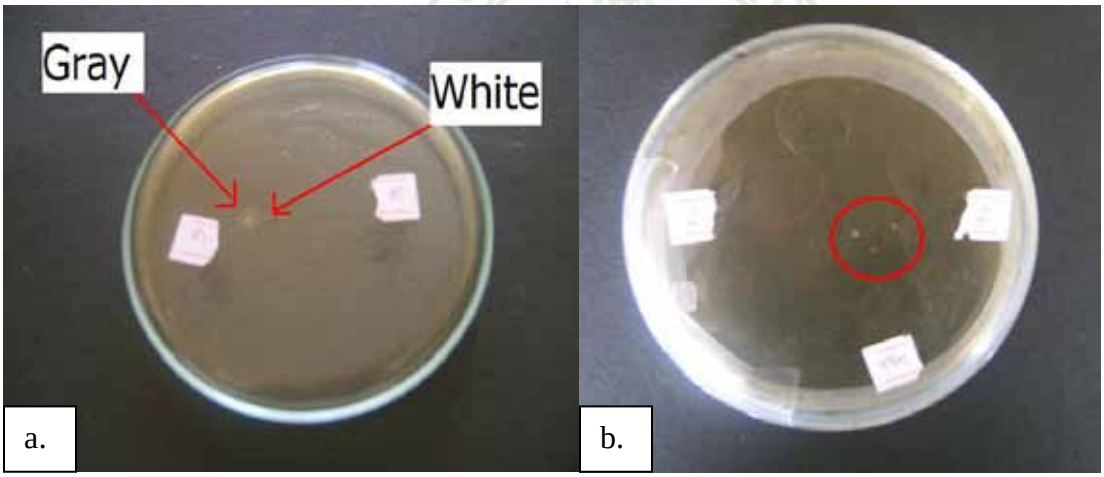

Figure 5. Plates showing growth of spore after 6 days from inoculation. (a) mycelium is observed from spore punch of *P.ostreatus* (Gray) while (b) no growth is observed from spore punch of *P. cornucopiae.*

A second set of single sporing was done this time pairing *P. ostreatus* spores but from different fruiting bodies. After 15 days from inoculation, mycelia from both spores were seen growing towards each other but only up to a certain point where one became inhibitory to the other thus the formation of an inhibition zone. This result is supported by

Holliday's (2004) findings on *Cordyseps sinensis*. He stressed that not all sub-strains within a strain will fuse, some are completely incompatible.

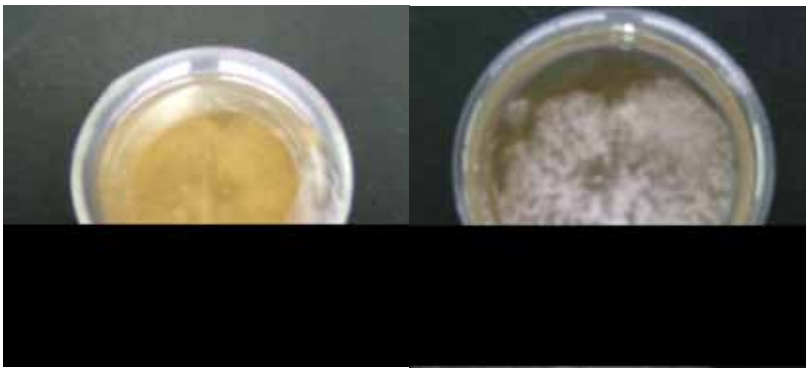

Figure 6. Formation of inhibition zone after 15 days from isolation.

Exposure Time to Ultra Violet (UV) Light

To increase the chance of hybridization, before subjecting it to UV light, five isolates from five single spores were transferred into a Petri dish. Results show that regardless of the length of time exposed to UV light, no clamp connections were observed in all plates. Fig. 8.g reveals no evident inhibition zone. Subjecting it though to mycelium diameter measurement also indicates no dikaryotization that took place.

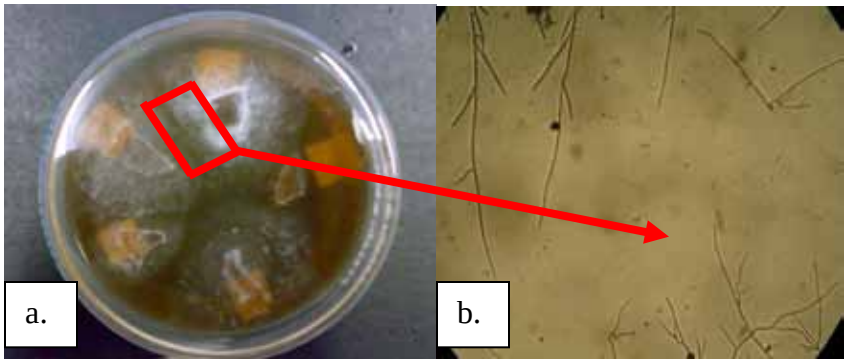

Figure 7. Monokaryons exposed to UV Light for 40 minutes (a) Inhibition zone between two different mycelia. (b) Zone of inhibition as seen under the microscope at 100x magnification.

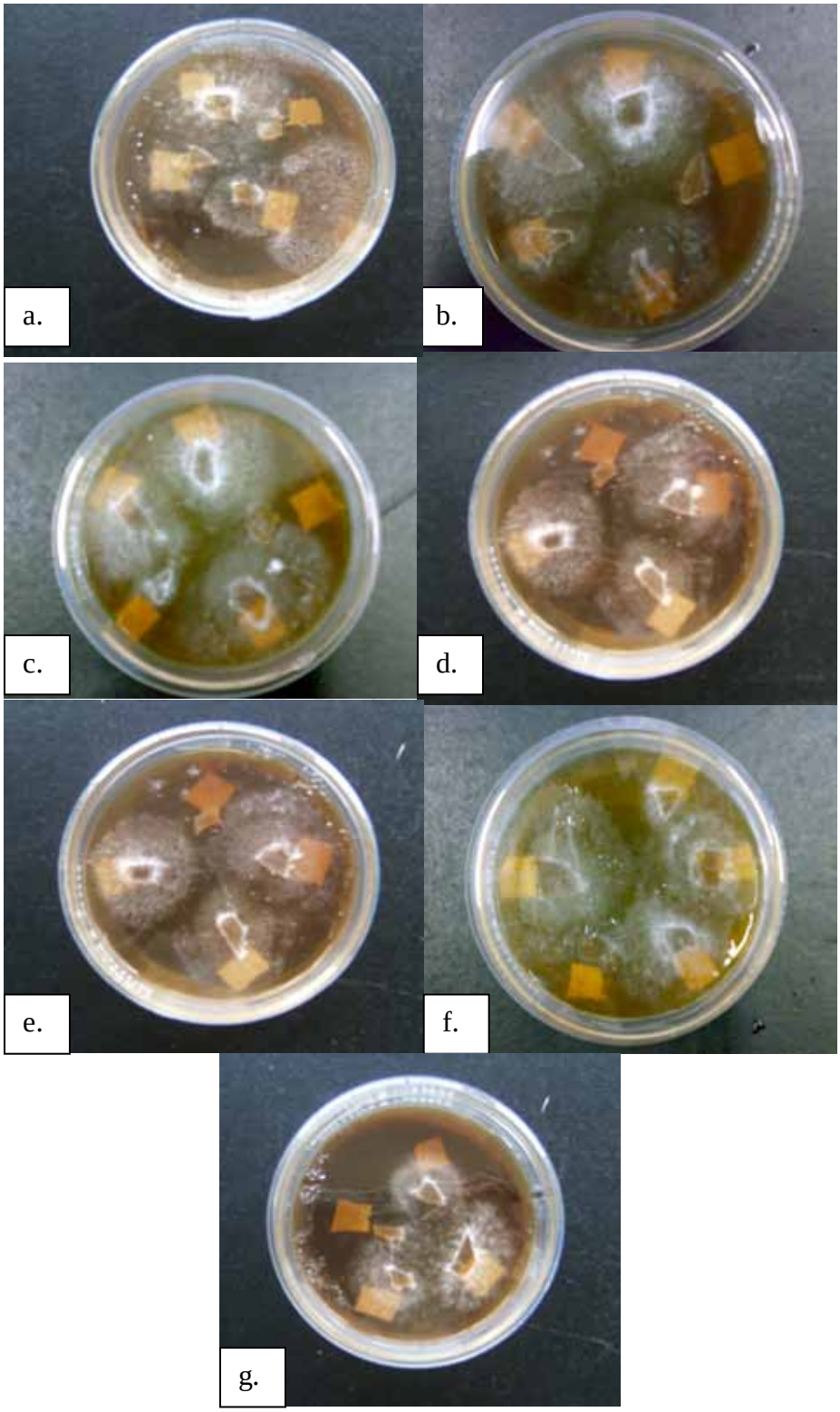

Figure 8. One week old culture of monokaryons exposed to Ultra Violet (UV) Light at different lengths of time (a) Control, (b) 10 minutes, (c) 20 minutes, (d) 30 minutes (e) 40 minutes (f) 50 minutes and (g) 60 minutes.

SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

Summary

This study was conducted to determine the kind of culture medium appropriate for spore germination, compare techniques for single sporing, determine length of time of exposure of germinated single spore to Ultra Violet light on dikaryotization and to determine for incidence of dikaryotic mycelium production.

Based on the results, malt extract agar is the most suitable medium for spore germination compared to potato dextrose agar and water agar. Although PDA and WA also sustain spore germination and growth, PDA produces shorter mycelium after two days from inoculation compared to MEA while water agar takes a longer time to be able to produce a germinated spore.

Spore punch method offers higher chances of obtaining single spores and is less laborious than that of the agar block method where the chances of obtaining a well isolated spore is slim.

Single spores obtained were paired together and were exposed to Ultra Violet light at different length of time. Regardless of this, no clamp connection was observed from these paired isolates.

Mycelia from both parents were compared to that of the supposed dikaryotic mycelium. These were measured but no difference in mycelial diameter was observed among the parents and the supposed hybrid.

Conclusions

Based on the findings of the study, it could be concluded that:

1. Among the media used, Malt Extract Agar is the most suitable medium for *Pleurotus spp*. spore germination and growth.

2. Spore punch method is the best technique to be able to obtain a well isolated single spore. It is much less laborious and requires shorter time of obtaining a single spore compared to the agar block method.

3. Regardless of Ultra Violet exposure at different lengths of time from 10 minutes to 60 minutes, dikaryotization did not take place, as observed in the plates and under the microscope.

Recommendations

Based on the findings, the following are recommended:

1. Further research need to be undertaken regarding growth of monokaryons in controlled setting among white oyster mushrooms.

2. Hybridization study on other mushrooms can be done with the given protocols on the kind of medium and single sporing technique from this study as a guide.

3. Other hybridization techniques including the use of mutagenic agents may be tested to improve the study.

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APPENDICES

Appendix Table 1. Effect of media and species on mycelial length

TREATMENT MEAN	REPLICATION I	II	III	IV	TOTAL
Media					
MEA 57.14	46.42	58.91	75.00	48.22	228.55
PDA 29.03	33.04	25.90	28.57	28.60	116.11
WA 0	0	0	0	0	0
Species					
P. ostreatus 27.82	25.00	32.74	22.62	30.95	111.31
P. cornucopiae 24.86	27.98	23.81	21.43	20.24	99.43

ANALYSIS OF VARIANCE

SOURCE OF VARIATION	DEGREE OF FREEDOM	SUM OF SQUARES	MEAN OF SQUARES	COMPUTED F	Significance	TABULAR F 0.05	0.01
Model	5	9447.077	1889.415	41.591**	.000	2.77	4.25
Species	1	119.483	119.483	2.630ns	.122	4.41	8.29
Media	2	9267.055	4633.527	101.997**	.000	3.55	6.01
Species x	2	60.538	30.269	0.666ns	.526	3.5	6.0

Media						5	1
Intercept	1	15721.985	15721.985	346.086**	.000	4.41	8.29
Error	18	817.704	45.428				
Total	24	25986.766					

ns = not significant
CV = 26.34%
** = highly significant