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WHITE OYSTER MUSHROOM (*PLEUROTUS FLORIDA*) MUTANT WITH ALTERED ANTIOXIDANT CONTENTS

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ABSTRACT

Radiation using gamma ray (60Co) at 0.75 KGray with dose velocity of 1.149 KGray/ hour on white oyster mushroom (*Pleurotus florida*) mycelia yielded several mutants. Based on isozyme analysis using two enzyme markers such as esterase (EST) and acid phosphatase (ACP) showed that 3 putative mutants (PO-3, PO-4 and PO-5) among 5 mutants are positive. Even though the isozyme patterns indicated that those 3 putative mutants are positively mutated, only PO-5 showed higher productivity compared to control (PO-K) which is reflected by significantly higher number of fruit bodies, higher fresh weight and dry weight yield of three successive flush periods. It was assumed that the mutation which occurred in PO-3 and PO-4 may affect other trait(s) of the white oyster mushroom. Antioxidant analysis of those mutants indicate that mutant PO-4 has significantly higher antioxidant content compared to control (PO-K) and the two other mutants (PO-3 & PO-5). This finding leads to the possible application of white oyster mushroom as a natural antioxidant source.

Key words : Mutant, white oyster mushroom, gamma ray, production, isozyme

INTRODUCTION

One way to introduce genetic variability is through mutation using chemical agents or ionizing radiation. Mutation may induce one or more change in characteristic of the mutated organisms. The characteristic changes may occur at the gene level which will be passed to the next generation (Carlile & Watkinson 1994). Mutation is applied to edible mushrooms to obtain better quality and productivity. The desired mutant characteristic can be economically beneficial for example resistant to pathogen, higher yield, etc.

There are two types of mutation based on the site in nucleus namely point mutation and chromosomal mutation. Mutation can alter the coding region or non-coding region. Mutation at coding region of a gene will cause the cell to experience silent, missense, frameshift and nonsense mutations. Mutation altering the non-coding

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region will cause different protein production or have no effect on mRNA maturation. Other types of mutation are null and leaky mutations. These two mutations occurred at the active site of a protein (Griffiths *et al.* 2000)

Gamma radiation is an ionizing radiation often applied to eukaryotic organisms. According to Esser (1971), gamma radiation is an effective ionizing radiation due to its ability to penetrate cell walls of mushroom mycelia. Gamma rays has higher energy which makes this type of radiation better penetrates into the target cells. Several sources of gamma rays are Cobalt-60, Cesium-137 and technetium-99 (Busby 2003). On eukaryotic cells, gamma radiation is able to break DNA molecules and change the purine and pirimidine bases of the target DNA (Kaiser 2001). In several microbiology studies, mutation that produced higher metabolites are mostly obtained by radioisotope mutagenesis (Slater 2000).

Ionizing radiation has been a common practice for extending storage life in mushrooms especially champignon. Radiation at 1.5 – 3 KGy on champignon fruit bodies will increase storage life from 8 weeks to 12 weeks (Maha & Pangerteni 1989). Ionizing radiation has also been used to generate mutants in several edible mushrooms. Elliot (1982) used ultra violet (UV) radiation to produce an *Agaricus bisporus* mutant strain that is resistant to fungicide. In this case, there is a possibility to produce mutant of white oyster mushroom (*Pleurotus florida)* that has higher productivity through ionizing radiation treatment.

White oyster mushroom (*Pleurotus florida*) is an edible mushroom that gained popularity lately due to its nutritional values and ease of cultivation. This mushroom contains higher amount of protein, lipid, phosporous, iron, thiamine and riboflavin compared to other edible mushrooms. In addition, this mushroom also contains 18 essential amino acids such as isoleucine, lysine, methionine, cysteine, phenylalanine, tyrosine, tryptophan, valine, arginine, histidine, alanine, aspartic acid, glutamic acid, glycine, proline and serine (Djarijah & Djarijah 2001). According to Bobek *et al.* (1989), oyster mushroom also rich in antioxidant makes this type of edible mushroom as an excellent source of natural antioxidant. The demand for natural antioxidant sources by food industries increased in the last decade due to the latest findings that indicated the harmful effect of the commonly used synthetic antioxidants such as BHA (butyl hydroxy anisole) and BHT (butyl hydroxy toluene). BHT and BHA are correlated to the damage of liver (Namiki & Osawa 1981). However, the antioxidant content of natural resources is very low. Therefore, it will be beneficial to obtain a white oyster mushroom strain that produces higher amount of antioxidant to fulfill the food industry demand.

One step to overcome this problem is to apply radiation in the form of gamma rays (60Co) to create mutants with commercial characteristics such as higher antioxidant content and many others. This experiment is intended to apply gamma radiation to white oyster mushroom to obtain genetic variability which may lead to commercially superior white oyster mushroom strains.

MATERIALS AND METHODS

The mutation material used in this experiment was white oyster mushroom (*Pleurotus florida*) local strain called TP. Media used for growing the mycelia were PDA (Potato Dextrose Agar) and PDB (Potato Dextrose Broth). All the mycelia were grown in PDA and PDB media at room temperature (± 28°C). Differences in mycelia diameter were observed on mycelia grown in PDA media while mycelia samples for isozyme analysis were grown in PDB media. Mutant and control were grown on substrates composed of saw dust (76.3%), rice husks (18.5%), chalk (2.96%) and gypsum (1.27%) as sugested by Chang & Miles (1989) for productivity observation.

Chemicals used in this experiment were chloramphenicol as an antibiotic which was added to media while ethanol 70% and 96% were used for sterilization. Chemicals used for isozyme analysis were liquid N, potato starch, ascorbic acid, cysteine, triton-X-100, PVP-40, $Na_2HPO_{4}.{}_2H_2O$, histidine monohydrate, citric acid monohydrate, TRIS hydoxylmethyl aminomethane, sodium phosphate, 1-naphtyl acetate, 2-naphtyl acetate, fast blue RR salt, sodium acetate, $CaCl_2$, H_2O_2 3%, 3-amino-9-ethylcarbasol, bromophenol blue, NAD, malic acid, NBT, PMS, TRIS-HCl, Na-1-naphtyl phosphate acid, fast garnet GBG salt and aquadest.

Approximately 0.5 X 0.5 cm^2 mycelia grown on PDA media were transferred to the fresh PDA media for radiation and some were reserved for stock. The same amount of mycelia (with and without radiation) were transferred to the PDB media for isozyme analysis. All the transfer and multiplication activity were done in the laminar air flow cabinet according to Djarijah & Djarijah (2001). Radiation procedure was done according to Esser (1971) with a radiation dose of 0.75 KGray using radiation velocity at 1.149 KGy/hour. Radiation was applied to mycelia grown on PDA media at exponential phase which is approximately 10 days after inoculation (Djajanegara *et al.* 2004). Growth curve of the white oyster mushroom (*Pleurotus florida*) used in this experiment was based on previous experiment by Djajanegara *et. al.* (2004). Isozyme analysis using acid Esterase and Acid Phosphatase were done according to Wendel & Weeden (1989). Antioxidant analysis was carried out by DPPH method (Yamaguchi *et al.* 1998) using BHA (butyl hydroxy anisole) as a standard.

RESULTS AND DISCUSSION

Gamma rays radiation was done on mycelia culture occupying approximately a quarter of the agar plate (10 days after inoculation). At 12 days after inoculation, the mycelia of white oyster mushroom were in the exponential growth period (Djajanegara *et al.* 2004). Just like any other edible mushroom, the white oyster mushroom has an extensive growth pattern as long as substrate is available. Mushroom cells are composed of vesicles excreting many types of ezymes and polymers to support the growth at the tip of hyphae. Hyphae cells at the tip of mycelium have high metabolism rate. Division on

those cells are extensive to produce new nuclei for the newly formed cell compartments (Alexopoulus *et al.*1996). Radiation application on those cells during that stage will be expected to produce maximum effect due to maximum radio-sensitivity of the highly metabolic rate of the undifferentiated cells (Prassad 1999).

There are a number of publications on plant improvement using mutation. This is in contrast with strain improvement in cultivated mushroom especially the oyster mushroom which cultivation has been recently introduced.

In order to verify the mutation, isozyme analysis was conducted. Isozyme which is also known as multiple molecular forms of enzymes is defined as enzymes that share a common substrate but differ in electrophoretic mobility (Markert & Moller 1959). They are revealed when tissues extracts are subjected to electrophoresis in various types of gels and subsequently submersed in solutions containing enzyme-specific stains This type of genetic analysis may indicate that some of the variant electromorphs are encoded by alternate alleles at a single locus, in which case the allelic products are termed allozymes (Prakash *et al.* 1969). The data in isozyme analysis show number and relative mobilities of various enzyme products when combined with appropriate genetic analysis become transformed into single or multilocus genotypes for each analyzed individual.

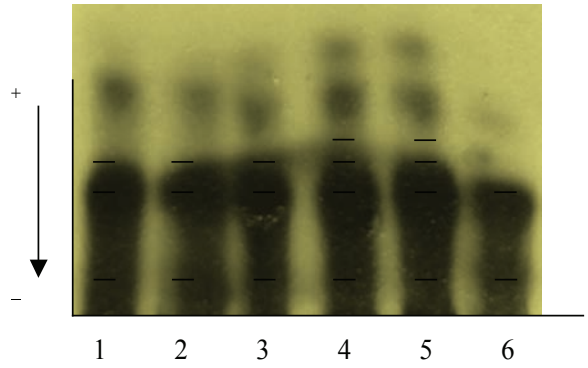


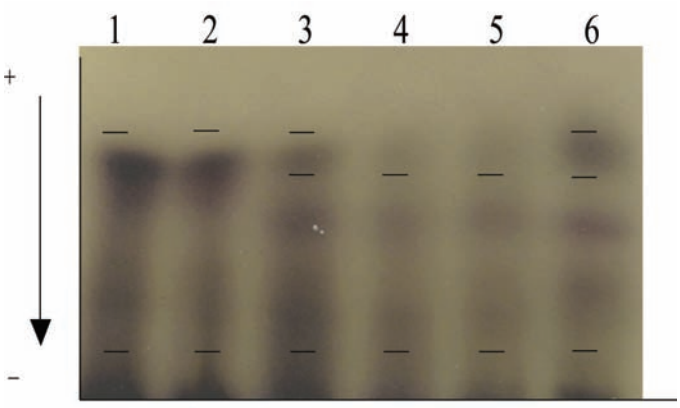
Figure 1. Starch gel of *Pleurotus sp.* assayed for Acid Phosphatase activity.

Notes :

1) Control (PO-K), 2) PO-1, 3) PO-2, 4) PO-3, 5) PO-4, 6) PO-5

Manifestations of mutation range from changes in intensity or relative intensity of bands to appearance or disappearance of bands (Tyson *et al.*1985). In this experiment, enzyme specific stains used are Acid Phosphatase (ACP) and Esterase (EST) (Figures 1 & 2). According to de Charisey (1985), subcellular localization of ACP is various in the cell with 2 – 4 numbers of isozymes. On the other hand, subcellular localization of EST is at the cytosol with 2 – 10 numbers of isozymes (Wehling & Schmidt-Stohn 1984). Isozyme analysis using those two enzyme markers will cover any mutation which occurs in the cell.

Figure 2. Starch gel of *Pleurotus sp.* assayed for Esterase activity.



Notes :
1) Control (PO-K), 2) PO-1, 3) PO-2, 4) PO-3, 5) PO-4, 6) PO-5

Isozyme analysis using Acid Phosphatase (ACP) as enzyme marker in this experiment showed 2 - 4 isozymes (Charisey 1985). In this experiment, we observed 3 bands in the control of wildtype white oyster mushroom using Acid Phosphatase (ACP) as enzyme marker which is in agreement with the reference. Those 3 bands constantly appeared in the zymogram of the wild-type white oyster mushroom sample as repeated 4 times (data not shown). There was no difference in the isozyme pattern of putative mutants PO-1 and PO-2 compared to control (PO-K). However, there was 1 additional band present in putative mutant PO-3 and PO-4 compared to control , while in the putative mutant PO-5 the top band that was present in control was not observed. Based on this analysis the mutation occurred in PO-3, PO-4 and PO-5.

In order to be certain with the mutants, another isozyme analysis using different enzyme marker was done (Fig. 2). Isozyme analysis using Esterase (EST) specific stain was chosen to verify the gamma ray mutation. Zymogram pattern of enzyme Esterase (EST) revealed 2 – 3 bands which is in agreement with the observation reported by Wehling & Scmidt-Stohn (1984). There was no difference between putative mutant PO-1 and control (PO-K). However, there was a different isozyme pattern between control and putative mutants PO-3 & PO-4 in which the relative migration of the top band was shifted. In the putative mutant PO-2 and PO-5, additional band was observed when the pattern was compared with control (PO-K). Interestingly, isozyme analysis using Acid Phosphatase enzyme marker on the putative mutant PO-2 did not show any mutation event but when the same sample was analyzed using Esterase enzyme marker mutation event was observed. Differences of the enzyme marker systems such as sub-cellular localization may contribute to this observation. Zymogram pattern of Esterase (EST) revealed that mutation occurred in PO-2, PO-3, PO-4 and PO-5.

However, based on 2 zymogram patterns of Esterase (EST) and Acid Phosphatase (ACP), it could be concluded that the mutants are PO-3, PO-4 and PO-5.

The manifestation of the mutation was investigated further by growth and morphological observations. There are no morphological differences between the mutants (PO-3, PO-4 and PO-5) and control (PO-K) in term of the shape and color of the fruit bodies. Data on productivity were reflected by amount of fruit bodies/bag log, diameter of fruit bodies, fresh weight (g) harvest/1 kg media (1 bag log) and fresh weight (g) of harvest/1 kg media (1 bag log). All the bag logs were treated equally and opened for aeration at the same time. Harvests were conducted at 3 successive flushing periods.

Based on productivity observation, only mutant PO-5 showed significantly higher productivity compared to control (PO-K) (Table 1). Biological efficiency ration (BER) of mutant PO-5 which is reflected by fresh weight of harvest/weight of media was higher than 10% for each harvest. The BER observed for PO-5 mutant was 21% (10 weeks post-inoculation), 14% (12 weeks post-inoculation) and 10.5% (14 weeks post-inoculation), while for the wild type were 10% (10 weeks post-inoculation), 8% (12 weeks post-inoculation) and 6.5% (14 weeks post-inoculation, respectively. According to Aryantha & Rachmat (1999), by reaching biological ratio efficiency at least 10%, the wood mushroom cultivation will be categorized as economically feasible. The mutant PO-5 also showed faster flushing period (4 days after aeration) compared to control (2 weeks after aeration). In this case, mutant PO-5 is a very useful strain to increase production.

Table 1. Growth performance of the white oyster mutants (PO-3, PO-4 & PO-5) compared to control (PO-K)

Types of white oyster mush-room	Number of fruit bodies/bag log			Diameter of fruit bodies (cm)			Fresh weight harvest (g)/ 1 kg media (1 bag log)			Dry weight harvest (g) / 1 kg media (1 bag log)		
	10 wpi	12 wpi	14 wpi	10 wpi	12 wpi	14 wpi	10 wpi	12 wpi	14 wpi	10 wpi	12 wpi	14 wpi
Control (PO-K)	28a	26a	12a	7.5a	6.8a	7a	100a	80a	65a	13a	10.4a	8.5a
Mutant PO-3	27a	26a	11a	8a	7a	7a	98a	78a	65a	12.7a	10.1a	8.5a
Mutant PO-4	28a	27a	12a	7.1a	6.5a	6.8a	97a	77a	67a	12.6a	10a	8.7a
Mutant PO-5	50b	42b	28b	9ab	9.6ab	9.5ab	210b	140b	105b	27.3b	18.2b	13.7b

Notes :

wpi = weeks post- inoculation to the bag log, a & ab = growth performance was not significantly different between genotypes based on t-test ($P>0.05$), b = significantly different ($P<0.05$)

Further analysis on the mutants (PO-3, PO-4 and PO-5) showed that mutant PO-4 has significantly higher antioxidant content in the fruit body compared to control (PO-K) and other mutants (PO-3 and PO-5). Antioxidant activity of the mutant PO-4 is equal to antioxidant activity of 47.627 mg/g BHA (Table 2). Higher antioxidant activity was always correlated with higher antioxidant content (Namiki & Osawa 1981). As a comparison, the antioxidant content of soymilk is equivalent to 29.5mg BHA/g while fermented soybean is equivalent to 72.8 mg BHA/g (Shahidi 1997).

Table 2. Antioxidant content of the white oyster mutants (PO-3, PO-4 & PO-5) compared to control (PO-K)

Types of white oyster mushroom	Antioxidant content (equivalent to mg/g BHA)
White oyster mushroom control (PO-K)	17.184a
White oyster mushroom mutant PO-3	16.945a
White oyster mushroom mutant PO-5	16.772a
White oyster mushroom mutant PO-4	47.627b

Notes:
a & ab = antioxidant content was not significantly different between genotypes based on t-test (P>0.05)
b = significantly different (P<0.05)

Several diseases are caused by strong oxidants such as cardiovascular disease, cancer and many others which can be inhibited by the use of antioxidants. Most of the damaging actions of oxidants are from reactive oxygen species (ROS) such as free radicals. Free radicals can be produced by dust, pollution, or as a side product of metabolism. Antioxidant is a chemical that is able to react with oxidant to inhibit the oxidation of bio-molecules (Langseth 1995). In addition, antioxidant is also required in food industry as a substance that is able to prevent lipid oxidation which leads to decay of food. Lipid oxidation is the first step for other changes in food that will have impact on nutrition value, food safety, color, flavor and texture of food (Shahidi 1997). In this case, the mutant PO-4 has a potential to be used commercially in the food industry as a natural antioxidant source.

CONCLUSIONS

Mutation using gamma rays (60Co) on white oyster mushroom (*Pleurotus florida*) yielded 4 putative mutants which are PO-2, PO-3, PO-4 and PO-5. Isozyme analysis using Acid Phosphatase (ACP) and Esterase (EST) as marker enzymes confirmed that mutation occurred at putative mutants PO-3, PO-4 and PO-5.

There were no differences in the morphology of the mycelia and fruit bodies between those mutants and control. Observation on mutants PO-3, PO-4 and PO-5 showed that the productivity of PO-5 was significantly higher compared to control and

the two other mutants (PO-3 and PO-4). Antioxidant analysis showed that mutant PO-4 has a significantly higher antioxidant content compared to control (PO-K) and the two other mutants (PO-3 and PO-5). This leads to the possible application of this particular white oyster mushroom (*Pleurotus florida*) mutant as a source for natural antioxidant which is beneficial for medical and food industry.

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