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Original article

Genetic Improvement of White Button Mushrooms for Qualitative and Quantitative Traits

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Summary: In this experiment, Gamma radiation was used for induction genetic diversity in white button mushrooms for achieving genotype(s) with desirable traits such as high yield and quality. Gamma radiation at dose 0, 100, 200, 300, 400, and 500 Gy per hour in air from a Cobalt 60 source were conducted on spreading spores on culture medium at room temperature (28 ± 2°C) at the Iran Atomic Energy Organization. 20 variants were selected based on physiological and mycelium growth rate differences for each gamma dose (totally 100 variants). The variants were incubated in compost. The experiment was conducted in a completely random design with three replicates. ANOVA results showed that significant differences among variants for days to complete spawn running, days to pin production and fruit body harvest, number of fruit body, fruit weight, yield, dry matter, and activation of laccase and manganese peroxidase enzymes ($p<0.05$). The lowest time for pin production and fruit body harvesting was observed in GR55 and GR63. Seven variants had higher Fruit body weight and GR54 lower than control. The results showed that gamma irradiation generated variation in the final yield of the three-flash harvest. Six variants, GR3, GR4, GR9, GR61, GR74, and GR72 were produced about 30% significantly more yield than the control. The results showed that the diversity in the white button mushroom can be improved by using gamma radiation, and the variation will be useful for the development of future breeding programs.

Key words: Gamma radiation, mutation, substrate degrading enzymes, white button mushroom, yield

Introduction

An increase in agricultural crop production caused to increase in agricultural wastes in recent decades. Mushroom production is considered a valuable tool for recycling this huge amount of waste. Mushroom has also high nutritional and medical values in comparison with other crops. Therefore, the mushroom is gaining importance as one of the most cost-effective agricultural products. There are not significant differences between the white button mushroom (*Agaricus bisporus* Imbash.) and the other kinds of mushrooms regarding nutritional and medical attributes, but its global expansion and sales market which makes it distinctive in among other edible mushrooms (1, 2).

Despite its extended history of classical and modern production along with high economic value, less effort is done in mushroom breeding (3), the main reason for this is the life cycle of *A. bisporus* and a result of the low variation. In most of the commercial populations and wild species of the white button mushrooms, the life cycle includes secondary homothallism in which, two spores are produced in each basidium instead of four in the meiosis division. Each spore includes two different, but compatible nuclei are named heterokaryon, and each heterokaryon can produce its self-fertile mycelial network, and spores of different mushrooms rarely can cross, thus the possibility of producing recombination is very low (4).

Inducing mutation with irradiation methods is a way of creating variation. One of the best radiations for inducing mutations is Gamma radiation. In eukaryote cells, Gamma radiation can break the DNA molecules and make changes in target purine and pyrimidine bases. Cobalt 60, Cesium 137, and Technetium 99 are sources for Gamma radiation 5. 6). Like other crops, the use of mutation inducers has been reported in mushrooms. Rasouli *et al*. (7), identified and isolated a mutant strain (M24-1) unable to produce spores by UV mutation on the white button mushroom mycelium. Isolation of improved strains for morphology and yield is one of the purposes for mutation induction in edible mushrooms. Djajanegara and Harosyo (6) used Gamma-radiation at a dose of 0.75 KG and produced a mutant strain having more fruit bodies with higher pin diameter and fresh and dry weights. Rosnani *et al.* (8) showed that in low dose Gamma treated oyster mushroom (*P.sajor-caju*), the mycelial growth rate was slower than higher dose, and it can increase the number of fruit bodies. Sermkiattipong and Charoen (9), treated the mycelia of *Volvariella volvacea* using Gamma-radiation (0.25-1.5 KG) and observed that mutants had more fruit bodies and earlier than the main strain.

Another important goal for mushroom breeding is improving the substrate degrading ability and therefore decreasing the costs and higher efficiency of compost, which could be achieved by mutation (10). Mushrooms generate some lignin-degrading enzymes (laccases, lignin per-oxidases, manganese peroxidases, etc.), to accelerate the degradation of lignocellulosic substrates (11). Kim *et al* (12) had been evaluated 67 Gamma-radiation treated strains of *Pleurotus eryngii*, and 5 strains showed higher cellulose and laccase activity. In the present study, spores of the white button mushroom were treated by Gamma irradiation, and qualitative and quantitative attributes were evaluated in some variants. Some selected variants were evaluated, and superior variants were selected base on desirable traits.

Materials and Methods

Spore print

Fresh fruit bodies of A15 strain were selected before or at the pileus opening stage. The stipe was removed, and the pileus was sterilized. Then the veil was removed by a sterile scalpel and the pileus was placed on a sterile petri dish. The complex was covered by a sterile suitable dish and spore print was formed on petri dish after 48 to 72 hours.

Irradiation

Spores spread into potato dextrose agar (PDA) medium and then they were irradiated by Gamma-radiation. The spore suspension was prepared from the spore print by sterile distilled water. Every 1 ml of the suspension contains 104 spores (counted by a hemocytometer and diluted repeatedly to obtain the desired concentration). The suspension was shaken thoroughly, and 1 ml of this suspension was transferred to another tube containing 4 ml of sterilized distilled water. Likewise, the serial dilution of spore suspension was made four times, until 1 ml of the suspension contain 20-25 spores, then was spread on PDA. Irradiation was conducted at the Iran Atomic Energy Organization and was applied to Gamma-radiation at 0, 100, 200, 300, 400, and 500 Gy doses.

Spawn preparing, Spawning, and harvest

After irradiation, Petri dishes were placed in darkness and 25°C. One hundred survived spores were selected based on differences in their morphology and mycelia growth rate. Variants of GR2-GR21 for 100 Gy, GR22-GR41 for 200 Gy, GR42-GR61 for 300 Gy, GR62-GR81 for 400 Gy, and GR82-GR101 for 500 Gy were selected. Selected variants were cultured into the sterilized wheat grain, after the mycelia completely grow through the grain, spawn prepared and incubated in compost. The compost and the casing soil were purchased from Bahman Yad Agro-Industry Company-Ardabil and the spawning was done in 1-kilogram compost bags with 7 grams of spawn.

Environmental factors for spawn growth and development were adjusted on the temperature of 25 °C and humidity of 90%. Soil amending was done after completely mycelial growth, then after the mycelia were developed completely in casing soil, the sudden reduction of temperature to 15°C for 72h was done for inducing mycelia growth stop and development of

pin organs. Then the temperature was set at 18°C for pin stimulation and growth until harvest.

Measurements of Morphological and Physiological parameters

Mycelia growth, pinning, and harvest: The number of days to complete mycelial growth before casing soil was the first trait measured to estimate the mycelia growth rate. Days to pinning and harvest were recorded from casing soil to indicate early ripening variants at different growth stages.

Numb*e*r of fruit bodies, fruit weight, and dry weight: The harvested fruit bodies in the bags were recorded as the number of fruits per bag. Mean fruit body size was calculated by dividing the fresh weight (g) to the total number of all harvested fruit bodies (13). For measuring dry weights, fruit bodies were divided into 4-8 pieces, and placed in the oven for 48h at 75°C, after taking the samples out of the oven, the percentage of dry weight was calculated. One gram of dried and powdered fruit body was placed at 550 °C for 6 hours, and ash percentage was calculated. The yield was recorded as the fresh weight of fruits for each bag at three harvests (14).

Lacasse and manganese peroxidase: For assay the activity of extracellular lignin-degrading enzymes, two gram of each substrate was picked up after complete mycelial growth, using liquid nitrogen was completely ground, then 4 ml sodium acetate buffer (0.1 M and pH: 4.50) and centrifuged at 4°C and 12000 rpm for 20 min. 2 ml of the supernatant was separated as an extract for enzyme activity assay (14). Lacasse activity was measured according to Shin and Lee (15), with minor modifications based on ABTS oxidation as substrate (14). The spectroscopic assay was performed at 421 nm using Spekol 1500 spectrophotometer. The enzyme activity was calculated as U. ml-1, using the ATBS extinction coefficient of 36000 $M^{-1}cm^{-1}$.

Manganese peroxidase activity was measured according to Bonnen *et al*. (16), based on Guaiacol oxidation as a substrate. The spectroscopic assay was performed at 465 nm using Spekol 1500 spectrophotometer. The enzyme activity was calculated as U.ml-1, based on the Guaiacol extinction coefficient 12100 M-1cm-1.

Statistical Analysis for investigation of variants: To evaluate the variants' characteristics, statistical analysis was carried out as a completely randomized design with three replicates. Analysis of variance (ANOVA) and mean comparison with Duncan's new multiple tests were done using the SPSS var. 21 software. Cluster analysis was performed by Ward´s method.

Results and Discussion

Spawn running, pinning and harvest

The variants that irradiated by gamma-radiation showed significant differences in time required for spawn running, the first appearance of pins, and harvesting of the fruit bodies ($p< 0.05$) (Table 1). The spawn running period on the compost bags ranged

Table 1. Analysis Of Variance for Effects Of Gamma Radiation Mutation Induction On White Button Mushroom Spores In PDA Medium, for The Studied Traits.

Sources of variation	Mean Squares										
	D.F.	Days to mycelial growth	Days to pinning	Days to harvest	Number of fruit bodies	Fruit weight (g)	Yield (g)	Dry matter	Ash	Laccase activity	Manganese peroxidase activity
Treatment	100	2.96**	3.00**	2.2**	18.29**	5.6**	4.65**	0.72ns	0.84ns	.006**	0.008**
Error	202	0.16	0.47	0.43	3.96	1.39	830.05	0.879	0.739	0.001	0.001
C.V. (%)		3.99	5.65	3.46	11.44	6.49	9.5	12.71	9.44	12.46	14.61

ns: non-significant, **: Significant at 1% level of probability

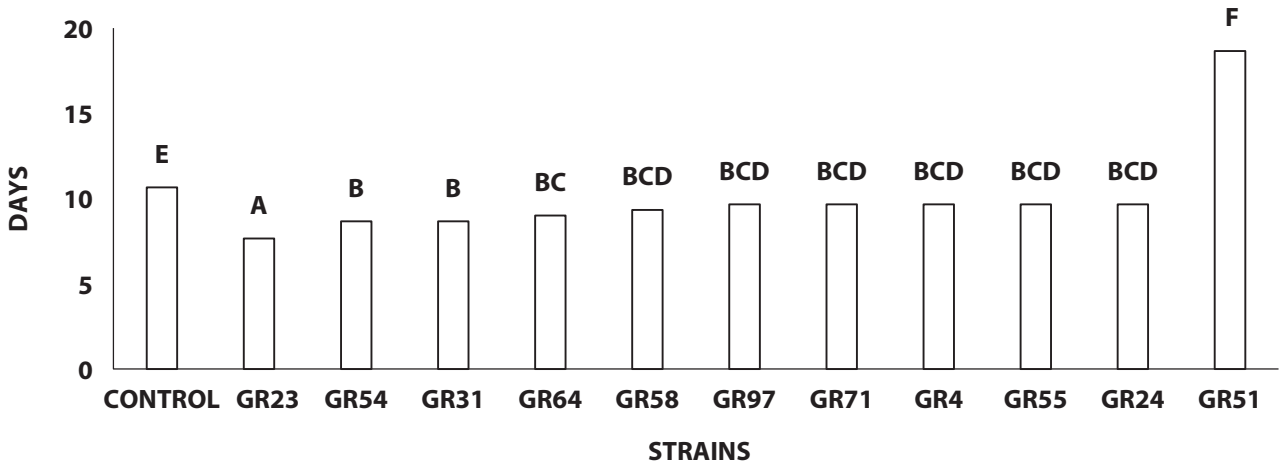

Figure 1. Mean of days from spawning to mycelia growth, in probably mutant strains isolated from gamma radiation treatments. **Different letters indicate significant difference* (ANOVA, *Duncan*, $p \leq 0.05$)

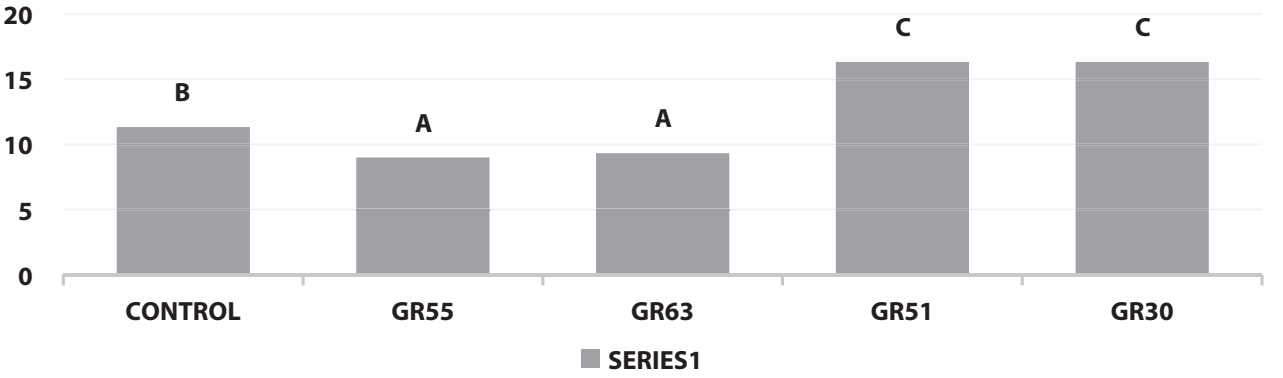

Figure 2. Mean of days from soil amending to pinning, in probably mutant strains isolated from gamma radiation treatments. **Different letters indicate significant difference* (ANOVA, *Duncan*, $p \leq 0.05$)

from 7.66-18.66 days in different variants. The minimum time to spawn running belonged to GR23. GR24, GR4, GR31, GR54, GR55, GR58, GR64, GR71, and GR97 that significantly had lesser time for spawn running than other variants. GR51 had significantly slower spawn running than the control (Figure 1). The range of time for pinning and fruit body harvesting was 9-16.33 and 16.33-22.66 days, respectively. GR55 and GR63 had a significantly earlier pining time than the control, but GR30 and GR51 reached a later time for pinning production (Figure 2). The lowest harvesting time was observed for GR55 and GR63 and the highest harvest time was GR51, GR57, and GR30 variants (Figure 3). Our results showed that gamma-radiation may cease genetic variations for life stages. Rosnani *et al.* (8) showed that in Gamma treated oyster mushroom (*P.sajor-caju*), the mycelial growth rate was slower than control, and it can increase the number of fruit bodies. Sermkiattipong and Charoen (2014), treated the mycelia of *Volvariella volvacea* using Gamma-radiation (0.25-1.5 KG) and observed that mutants had earlier than the main strain.

Number and fresh weight of fruit body and mushroom yield

Gamma irradiation was induced genetic variability for fruit body number, fruit body fresh weight,

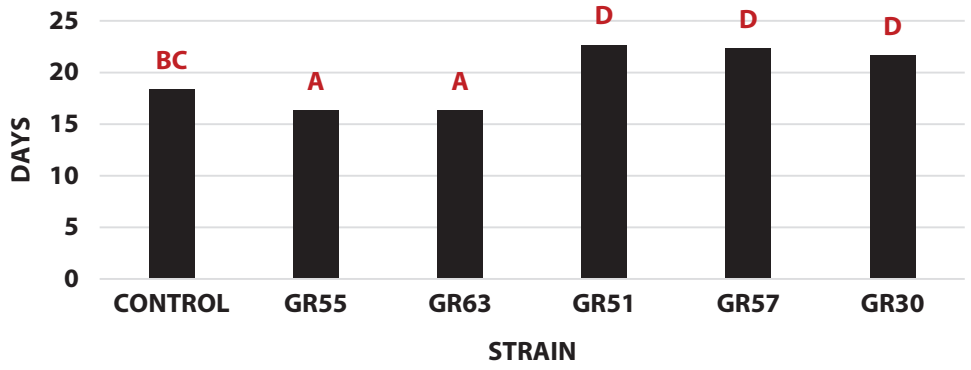

Figure 3. Mean of days from soil amending to harvest, in probably mutant strains isolated from gamma radiation treatments.
**Different letters indicate significant difference* (ANOVA, *Duncan*, p ≤ 0.05)

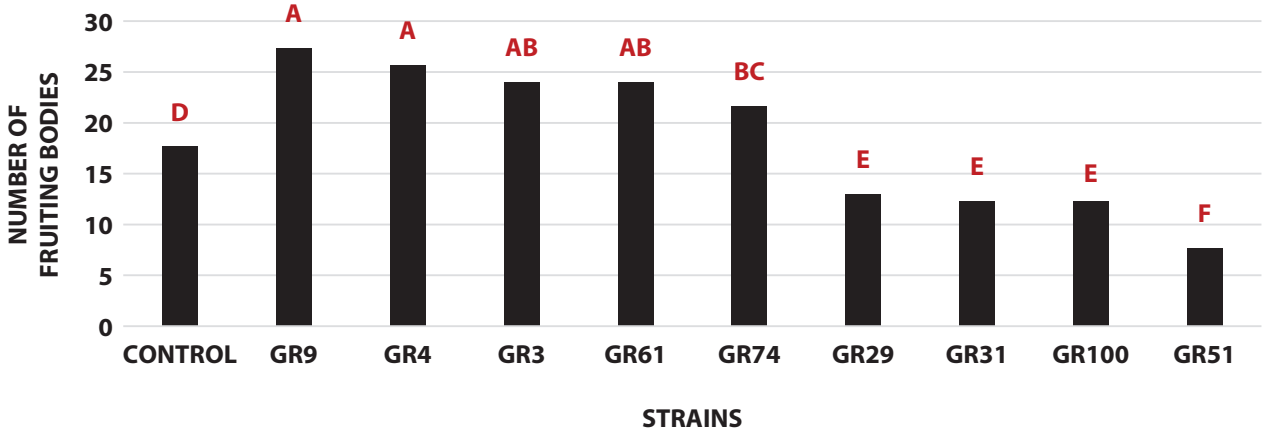

Figure 4. Mean of number of fruit bodies, in probably mutant strains isolated from gamma radiation treatments.
* *Different letters indicate significant difference* (ANOVA, *Duncan*, p ≤ 0.05)

and yield. The mean number of fruit bodies, fruit body weight, and final yield ranged from 27.33–7.6, 14.05 to 24.22 g, and 142.51-432.29 gr per one kg of the substrate, respectively. The results revealed that significant differences among variants for the traits ($p < 0.05$), but the difference among variants for dry weight was not significant ($p > 0.05$) (Table1). Five variants (GR9, GR4, GR3, GR61, and GR74) had a significantly higher number of fruit bodies than control, but there was no significant difference among these variants. The lower number of fruit bodies was observed in GR29, GR31, GR100, and GR51 (Figure 4). One of the most important components of yield is fruit body weight. The formation and development of the fruit body in fungi is the result of the integration of several environmental factors and is regulated by many genes and proteins (14). In this study gamma irradiation treatments significantly influenced fruit body weight. Seven variants (GR14, GR6, GR55, GR17, GR71, GR72, and GR63) had higher Fruit body weight and GR54 lower than control (Figure 5). Maximum fruit body weight observed in GR63. Yield is one of the most important traits in crops, which is affected by environment and genetic effects. The results showed that gamma irradiation generated variation in the final yield of the three-flash harvest. Six variants, GR3, GR4, GR9, GR61, GR74, and GR72 were produced about 30% significantly more

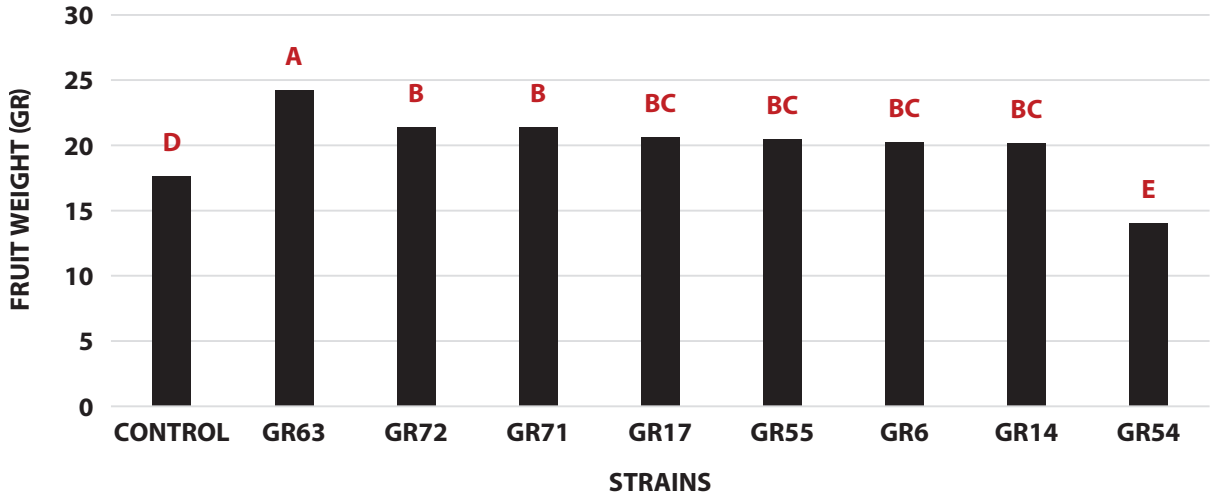

Figure 5. Mean of fruit weight, in probably mutant strains isolated from gamma radiation treatments.
**Different letters indicate significant difference* (ANOVA, *Duncan*, $p \leq 0.05$)

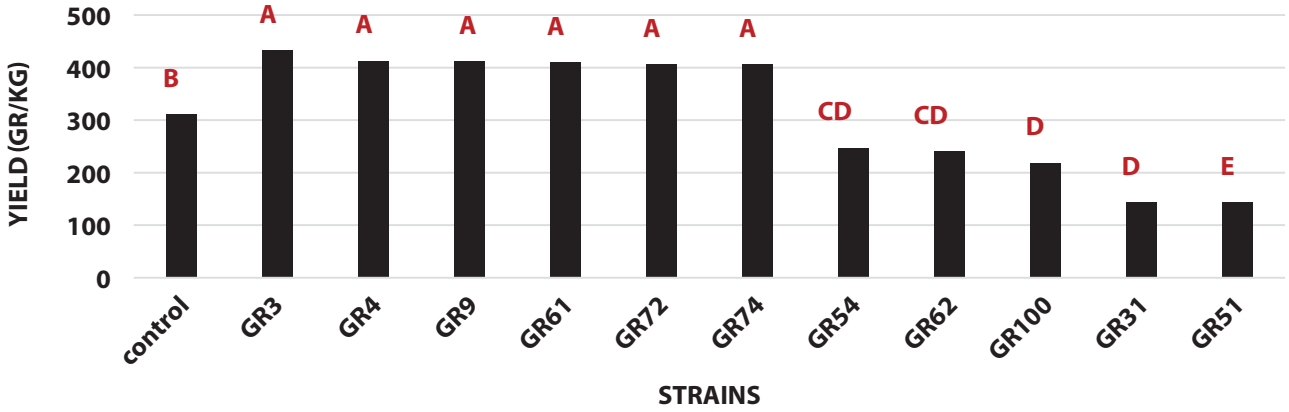

Figure 6. Mean of yield, in probably mutant strains isolated from gamma radiation treatments.
**Different letters indicate significant difference* (ANOVA, *Duncan*, $p \leq 0.05$)

yield than the control, however lower yield was observed in GR54, GR62, GR100, GR31 and GR51 (Figure6). The Minimum yield (142.51 g/kg substrate) was observed in GR51, and the Maximum (432.29 g/kg substrate) was observed in GR3. At first, the main purpose of this study was to create variation and then selection of variants with better yield, and the variants with higher and lower yields in comparison with control were successfully achieved. Rosnani *et al.* (8) showed that in low dose Gamma treated oyster mushroom (*P.sajor-caju*), increase the number of fruit bodies. Sermkiattipong and Charoen (9), treated the mycelia of *Volvariella volvacea* using Gamma-radiation (0.25-1.5 KG) and observed that mutants had more fruit bodies.

The variants with a higher number of fruit bodies showed higher yields such as GR72 with higher fruit body weight resulted in a higher yield. Although GR63 showed a significant increase in fruit body weight, it did not affect the final yield. Based on our result, the number of fruit bodies is a more determining factor in yield than fruit body weight.

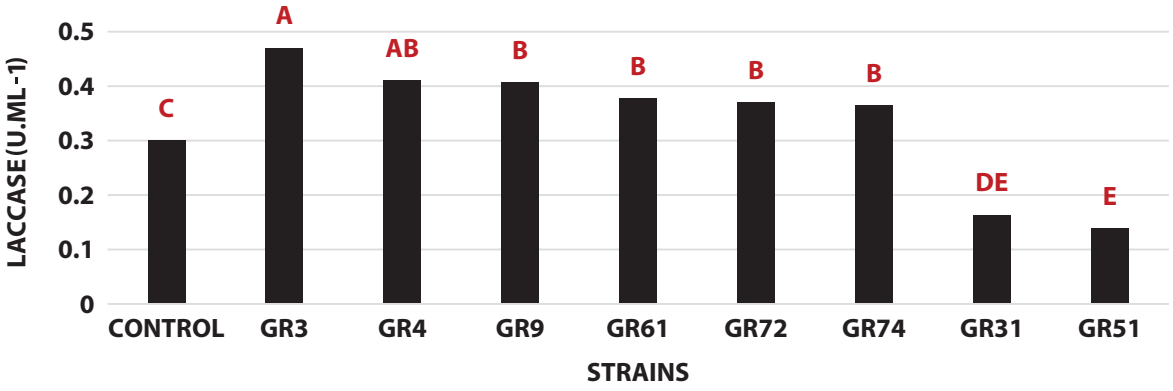

Figure 7. Mean of Laccase enzyme activity, in probably mutant strains isolated from gamma radiation treatments.
**Different letters indicate significant difference* (ANOVA, *Duncan*, p ≤ 0.05)

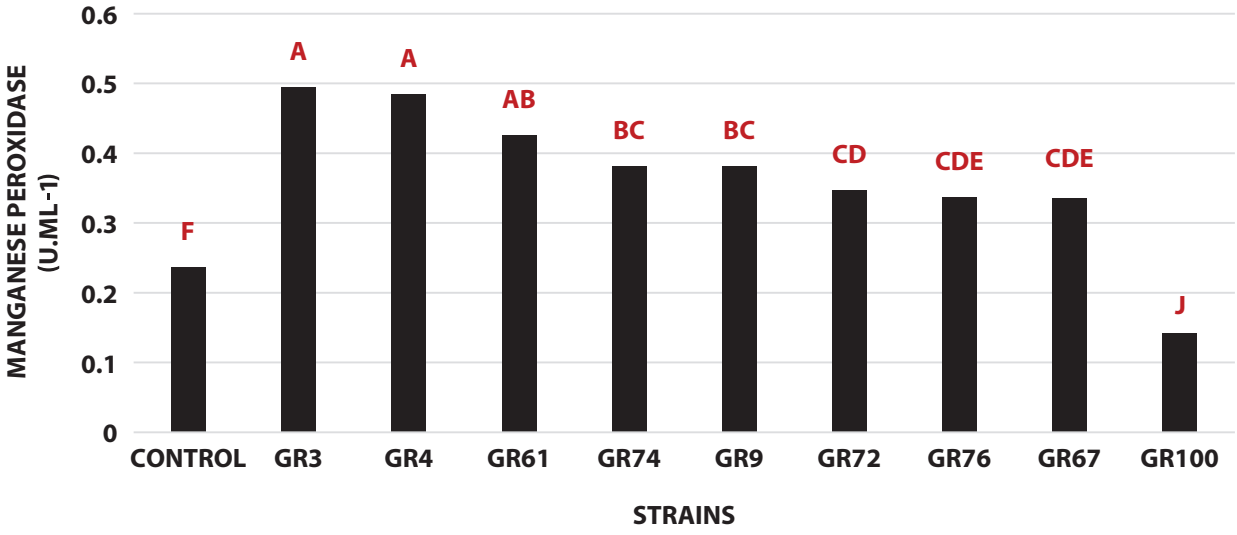

Figure 8. Mean of Manganese peroxidase enzyme activity, in probably mutant strains isolated from gamma radiation treatments.
**Different letters indicate significant difference* (ANOVA, *Duncan*, p ≤ 0.05)

Laccase and Manganese Peroxidase

In this study, genetic variation was observed for laccase and manganese peroxidase enzyme activities (p< 0.05) (Table1). Among variants GR3, GR4, GR9, GR74, and GR61 were showed significantly more laccase activity than the control, and GR3 had maximum laccase activity but two variants GR31 and GR51 had minimum enzyme activity (Figure 7). GR3, GR4, GR61, GR74, GR9, GR72, and GR67 had significantly higher activity of manganese peroxidase activity than the control. GR3 and GR4 had a maximum of manganese peroxidase enzyme activity and GR100 had minimum the enzyme activity (Figure 8). Kim *et al* (12) had been evaluated 67 Gamma-radiation treated strains of *Pleurotus eryngii*, and 5 strains showed higher cellulose and laccase activity.

During mycelium growth, lignin-humic-like substances are degraded, which is proposed to help the accessibility of carbohydrates in the compost during fruit body formation. Furthermore, a compost that is colonized to a further extent with mycelium is known to generate more fruit bodies in the following growth phase. Selection criteria should be the ability to metabolize lignin-/humic-like structures during mycelium growth and colonization abilities while

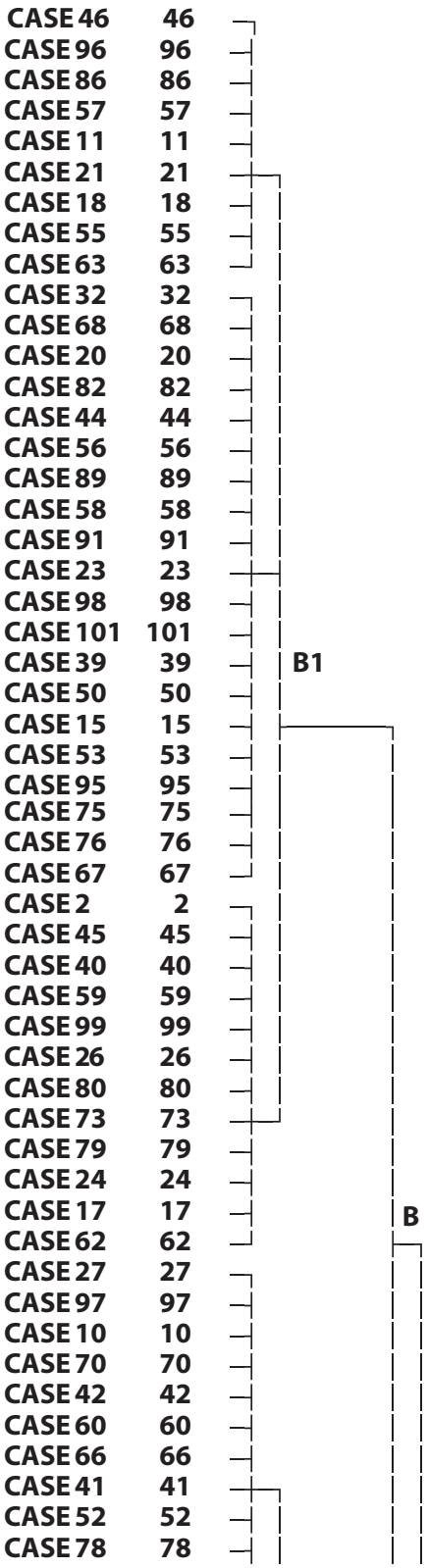

Figure 9. Cluster analysis of variants based on studied traits in first experiment, including yield, Laccase and Manganese Peroxidase activity.

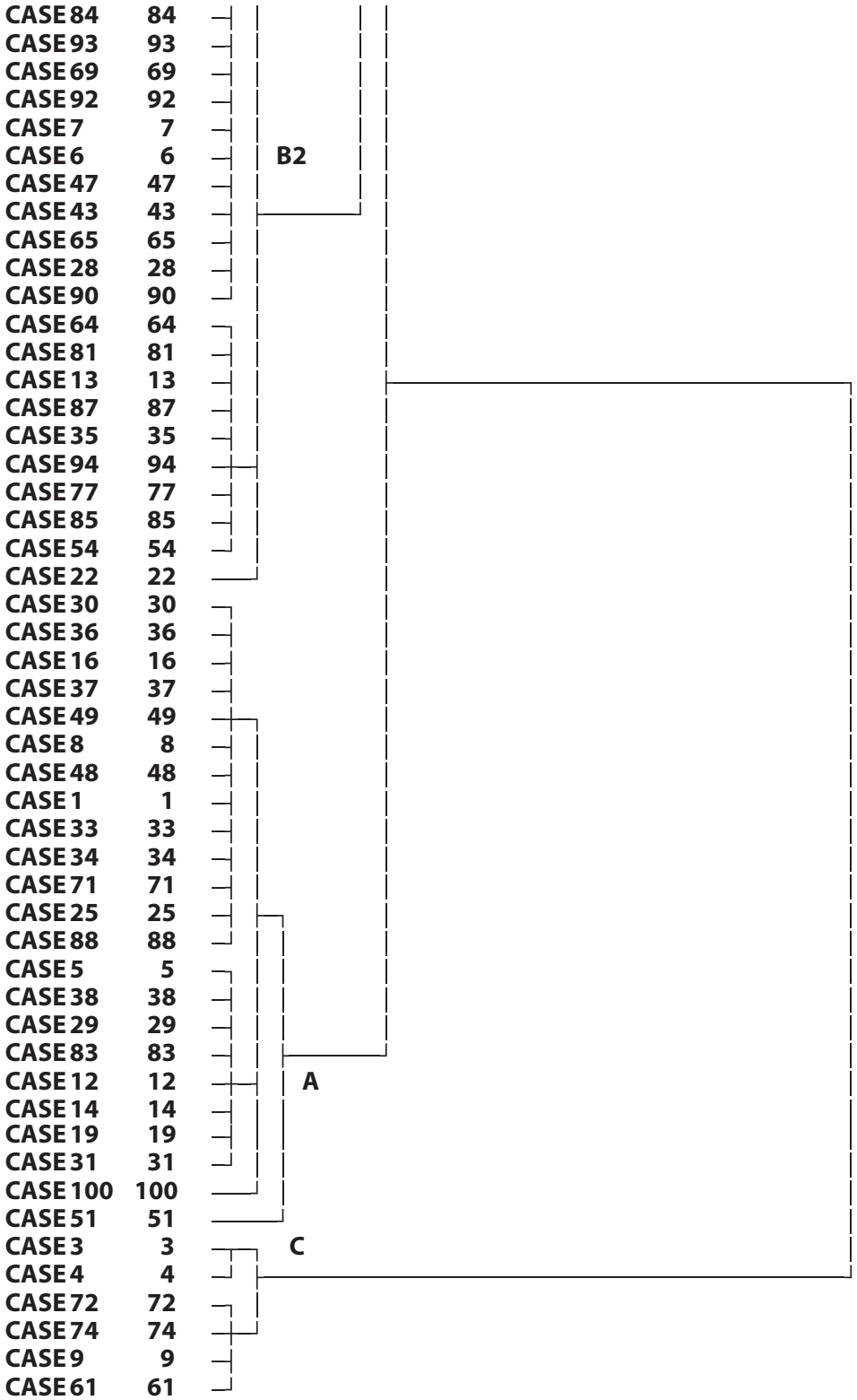

Figure 9. (Continued)

Table 2. Summary Of Cluster Analysis Based on Studied Traits In First Experiment Including Yield, Laccase And Manganese Peroxidase Activity

			Yield (g)		Laccase activity		Manganese peroxidase activity	
Group	Number of variants in group	Number of selected variants	Mean of group	Mean of selected variants	Mean of group	Mean of selected variants	Mean of group	Mean of selected variants
A	23	1	281.78	308.97	0.303	0.302	0.228	0.238
B1	41	6	303.26	327.41	0.296	0.312	0.302	0.297
B2	31	2	297.08	321.09	0.2480.401	0.171	0.270	0.276
C	6	6	413.3	413.3	0.3	0.401	0.419	0.419
Control	1	1	311.74	-	0.289	-	0.237	-
Total	101	16	303.01	-		-	0.282	-

maintaining the ability to produce commercial quantities of fruit bodies in the reproductive phase (7). The results indicated variants with high lignin-degrading enzyme activity caused to high final yield.

Selection of superior variants based on cluster analysis

Results of cluster analysis based on the mean of the traits showed that irradiated variants divide into four groups and superior variants were selected from each group with higher yield as well as lignin-degrading enzymes than the control and other variants. These superior variants included: GR33 from A group, GR55, GR63, GR67, GR50, GR96, and GR18 from B1 group, GR93, and GR22 from B2 group and GR3, GR4, GR9, GR61, GR72, GR74 from C group. The means of the groups showed that there were variants in group C which had maximum yield and the activity of substrate degrading enzyme (Table 2).

Conclusions

According to the lifecycle of the white button mushroom, thereby decreasing variability is the main obstacle in the breeding programs, so irradiation can be a useful tool for inducing genetic variation trough new mutants. Our result showed mutation by Gamma-radiation could be effective in creating diversity as an early step for classical and molecular breeding, in other words, the application of gamma-radiation treatments on the white button mushroom spores created desirable variation in the traits. It can also accidentally result in the isolation of a new strain. The variation of yield was observed along with changes in lignin-degrading enzymes activities, it can be said that the higher yield is probably due to enhancement in the enzyme activity.

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