Malays. Appl. Biol. (2017) 46(1): 77–82

VARIANTS PRODUCTION OF *Lentinula edodes* SPORES, MONOKARYON MYCELIUM AND DIKARYON MYCELIUM BY γ-IRRADIATION

IBRAHIM MAHMOOD[1,2], AZHAR MOHAMAD[2], FAUZI DAUD[1], ZAITON ABDUL KADIR[1,2], HING JAN NIE[2], HISHAM HAMZA HUSSAIN[1,2], WAN SAFINA WAN ABD RAZAK[1,2], AIRIANAH, O.B.[1] and SHAZRUL FAZRY[1*]

[1]*School of Biosciences and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 Bangi, Selangor, Malaysia*
[2]*Malaysian Nuclear Agency, Bangi 43000, Kajang, Selangor, Malaysia*
[*]*E-mail: shazrul@ukm.edu.my*

Accepted 30 January 2017, Published online 31 March 2017

ABSTRACT

Lentinula edodes or commonly known as shiitake is a cultivated edible mushroom. This mushroom has been used in pharmaceutical, nutraceutical and cosmeceutical industries. However after years of selective inbreeding, the mushroom suffers from a small genetic pool. In this study, we attempt to produce genetic variants of shiitake via γ-irradiation. Shiitake's spores and mycelium (both monokaryon (MM) and dikaryon (DM)) were subjected to γ-irradiation (^{137}Cs) at series of doses ranging from 0 to 4000 Gy. Dose responses were evaluated based on mycelium growth performance and survivability. The γ-irradiation LD_{50} for shiitake's spores, MM, 3rd DM subculture and 9th DM subculture were 795 Gy, 460 Gy, 735 Gy and 1330 Gy, respectively. Interestingly our result shows those higher subcultures DM are more resistant to γ-irradiation. We observed that MMs which were exposed to higher dose of γ-irradiation show lower numbers of clamp connections compared to control indicating that there are genetic compatibility changes between individual hyphal cells within the irradiated shiitake mycelia. This study revealed the potential of γ-irradiation as a tool to create diversity in shiitake genetic pool towards healthy and sustainable mushroom crops in the industries.

Key words: gamma (γ) irradiation, *Lentinula edodes*, monokaryon mycelium, dikaryon mycelium, spores, shiitake

INTRODUCTION

One of the major issues plaguing commercial and cultivated mushrooms (especially *Lentinula edodes*) in the industries is the limitation of its genetic pool due to constant selective inbreeding (Chiu *et al.,* 1996; Xiang *et al.*, 2016). This has also made it difficult for farmers and the scientific community to breed new strains of mushrooms. It is vital that new strains of mushrooms should be developed to maintain a healthy and sustainability of these mushroom species in the future. The life cycle of the *L. edodes* (commonly known as shiitake mushroom) involves dispersal of spores, germination of single nucleus monokaryon mycelium (MM) (a branching network of hyphae, thread-like structures of the fungus cells), mating of monokaryon via clamps connection to form two nuclei dikaryon mycelium (DM), and eventually development of fruiting body (Alexopolous *et al.,* 1996; Souza-Paccola *et al.*, 2004). It is through these mating processes that ensure the dynamic of genetic diversity of any fungus. It is also should be noted that every mushroom has a unique complexity to achieve this mating process (Gola *et al.,* 2000).

Alteration in the structure of DNA sequence (also known as mutation) can be caused by physical or chemical agents known as mutagen (Crow & Abrahamson, 1997; Djajanegara, 2008). Previous studies have shown that mutation can be induced via radiation and diversity of crops can be created from these mutations (Stadler & Sprague, 1936a; Stadler & Sprague, 1936b; Crow & Abrahamson, 1997; Djajanegara, 2008; López *et al.,* 2016). γ radiation is able to penetrate cell walls and cause DNA double strand breaks, which increase the

* To whom correspondence should be addressed.

possibility of genetic alteration by faulty DNA repair. An example of desired mutation on mushroom via radiation has been previously shown by Djajanegara (2008) where white oyster mushroom (*Pleurotus florida*) which has been γ-irradiated produced higher number of fruit bodies and higher fresh and dry weigh compared to the controls. The aim of this study is to investigate the effect of γ radiation on *L. edode*s spores, MM and DM growth performance and survivability. The outcomes of this study will help to identify the LD_{50} of *L. edodes* for γ radiation (Lethality Dose 50, where the irradiation dose cause 50% death to the mushroom), which is important for future *L. edode*'s mutational work via γ radiation.

MATERIAL AND METHODS

Sample materials

Matured fruiting bodies of *L. edodes* were gathered from Kundasang, Sabah, Malaysia. The fruiting body was then capsized to produce spore print on a trace paper and stored at -20°C. MM was obtained by germinating the spores on a potato dextrose agar (PDA) (OXOID) at 25°C for 2 weeks. DM was cultured from a stem cut-off from the shiitake fruit body and grown on a PDA at 25°C for 2 weeks. The DM was then subcultured by transferring the mycelium (punch) onto a fresh PDA using a gel puncher. In this study, we have used mycelium that has been cultured three (3rd subculture) and nine (9th subculture) times for the irradiation experiments.

Dose of γ-irradiation

BioBeam GM8000 with ^{137}Cs as source was used to expose spores and both MM and DM to acute γ-rays. The doses ranged from 0, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1500, 2000, and 4000 Gy at a dose rate of 13.1 Gy/min. The calculation for LD_{50} was done as performed by Miller and Tainter in 1944 (Randhawa, 2009).

Identification of clamps connection

A small piece of carefully cut mycelium (without the agar) was mounted on a glass slide. Then, 40 µl of distilled water was mounted on the slides before covered with a cover slip for observation. A microscope (OLYMPUS) with 40× objective magnification lens was used to observe clamp connections.

Growth performance evaluation

To investigate the dose response of γ-irradiation on the growth performance of *L. edodes,* the diameter growth of the irradiated mycelium on PDA was measured as described by Ibrahim *et al* (2015) after 14 days. To summarise, the mycelium punch (0.7 cm in diameter) were place at the centre of the PDA and were grown at 25°C. At day 14, the diameters of the growing mycelium were measured from the two lines that cross the centre of the mycelium punch (Figure 1). The growth of the mycelium was calculated by averaging the diameters (cm) per the total number of days of the experiments.

RESULTS AND DISCUSSION

This study, show the effects of γ radiation on different stages of shiitake after 14 days (Figure 2.). We observed that the spores and 9th DM subculture have a higher lethal dose or resistant towards γ radiation, which are 1500 Gy and 2000 Gy, respectively compared to MM (800 Gy) and 3rd DM subculture (1000 Gy). We hypothesised that this may due to the structure of the mushroom during this stage, where they are more protected in the spores structure (Moeller *et al.,* 2012) and shielded by layers of hyphae formation at the 9th DM subculture which grow much faster compared to the 3rd DM subculture, reducing the effect of γ radiation (Figure 3).

It is also evident that prolong subculturing of mycelium will cause the mycelium to grow much stable and faster due to adaptability of the cells towards the growing media (Gow & Gadd, 2007; Chauhan & Jaswal, 2015). These results correlate with the LD_{50} of mycelium against γ radiation. The LD_{50} for each mycelium in this study are 795 Gy, 460 Gy, 735 Gy and 1330 Gy were spores, MM, 3rd

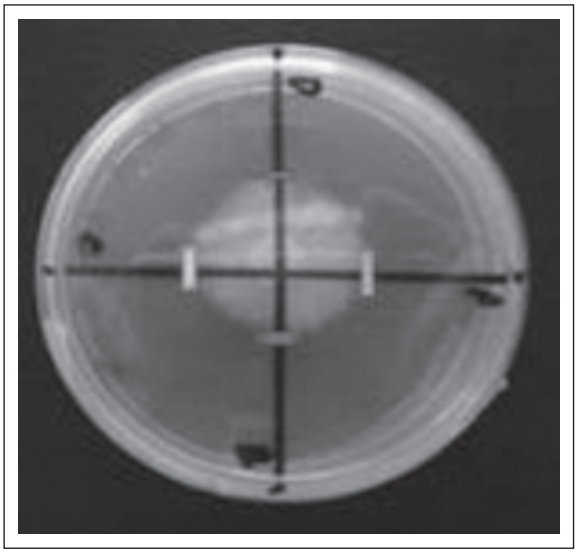

Fig. 1. Determination of growth rate and performance of γ-irradiated mycelium. 10 replicates were done for each experiment. The yellow and red lines indicate the range of where the diameters of the mycelium were measured.

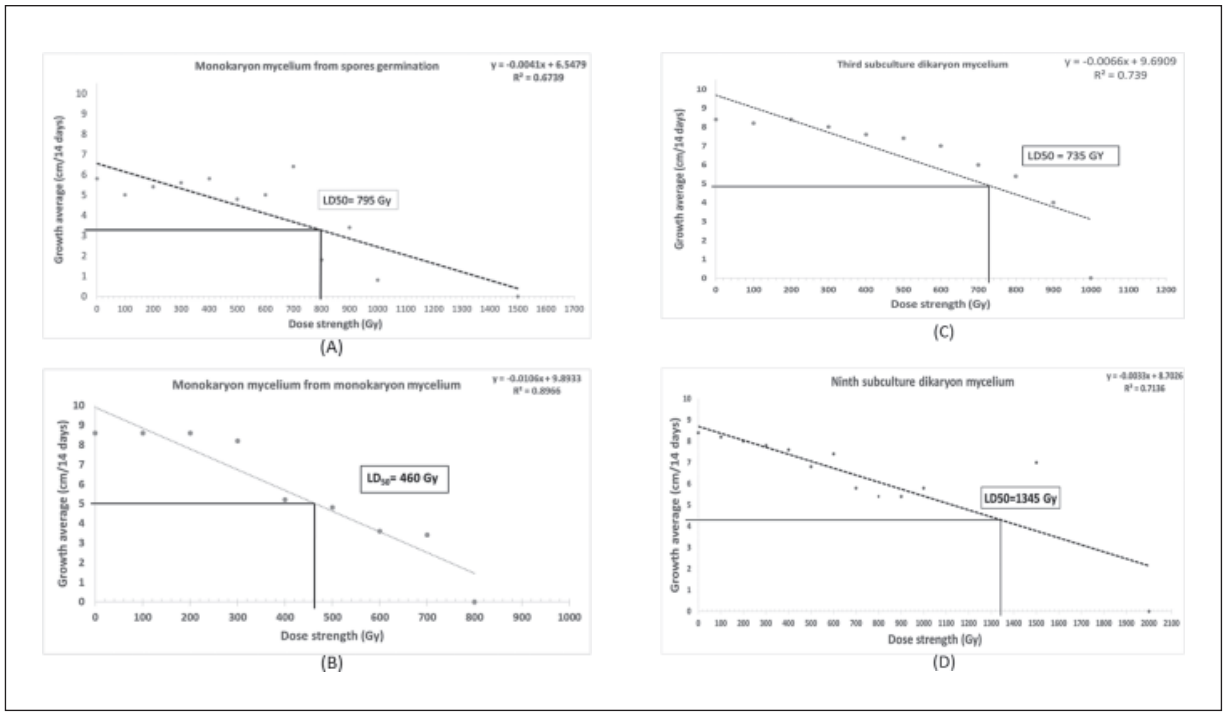

Fig. 2. Dose response of spores of different type of mycelium to γ-irradiation. **(A)** Spores, **(B)** MM, **(C)** 3rd DM subculture, and **(D)** 9th DM subculture. Growth rates after 14 days of mycelium samples after acute gamma irradiation at different doses.

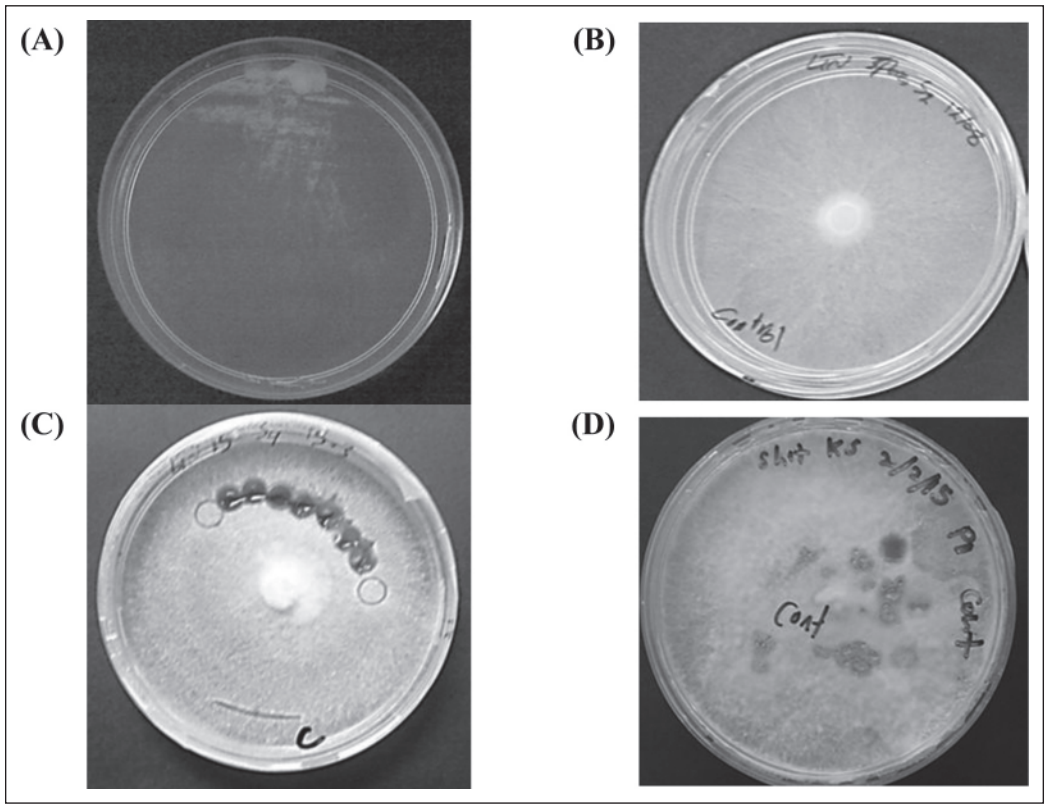

Fig. 3. Morphology of spores and mycelium. **(A)** Spores, **(B)** MM, **(C)** 3rd DM subculture, and **(D)** 9th DM subculture. Sources of mycelium used for the subculturing were from the punch holes which could be observed in **(C)**. The layers of mycelium in **(D)** are denser compared to the layer of white mycelium in **(C)**, which may contribute to its resistance to γ radiation.

DM subculture and 9th DM subculture, respectively. From here we speculate that the best γ-irradiation dose to create *L. edodes* mutant variants is at 700 Gy. To verify this speculation, spores and mycelium were exposed to 700 Gy of γ radiation and grown on PDA at 25°C for 14 days (Figure 4). The 4000 Gy dose was used as a negative control, since γ-irradiation of more than 2000 Gy in general are lethal to the mushroom. As expected the irradiated MM died after irradiation, however both DM (3rd subculture and 9th subculture) survive the irradiation treatment. More interestingly, MM germinated from the germinated spores (Figure 4B1) grows much faster compared to the control (Figure 4A1). This indicates that it is best to irradiate spores than mycelium to create new and more robust *L. edodes* variants.

Clamp connections are the combination of two hyphal cells that have high affinity (positive polarity) towards each other. MM shows a low tendency to form clamps because of its genetic composition (Xing *et al.,* 2016). The growth rate of a mycelium depends on the formation of clamps, which enables the exchange of genes and extension to more areas (Kothe, 2001). This observation was also observed by Rosnina *et al* (2016) when they show faster growth of compatible mated mycelia of wild *Pleurotus citrinopileatus*. The exposure to severe γ radiation decreases the number of clamps, which may due to the alteration of the genetic compatibility of these mycelium. The number of clamps for MM was 1-3 /HPF (High Power Field) and DM was 4-6/HPF. Figure 5 illustrates the reduced number of clamps form in MM culture proportionate to the increased of exposure to γ radiation.

CONCLUSION

We proposed that the best method to produce *L. edodes* mutants via γ-irradiation is by irradiating spores at 700 Gy. The numbers of clamp connections

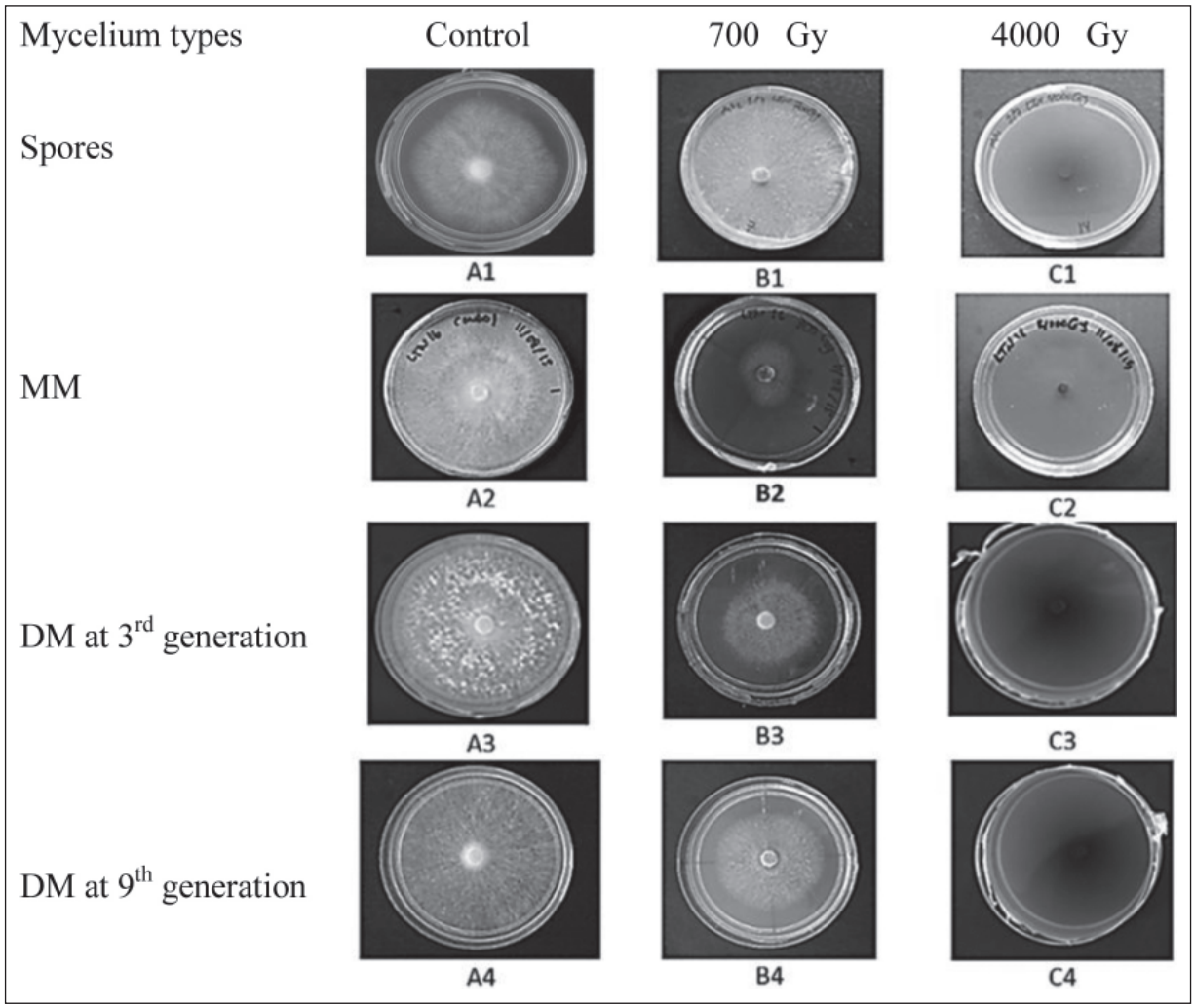

Fig. 4. Effects of γ-irradiation on the growth rate of mycelium. **A1, 2, 3 and 4** are the growth of the control mycelium after 14 days. **B1, 2, 3 and 4** are the growth of irradiated (700 Gy) mycelium after 14 days. **C1, 2, 3 and 4** are the growth of irradiated (4000 Gy) mycelium after 14 days, which cause lethality on the mycelium.

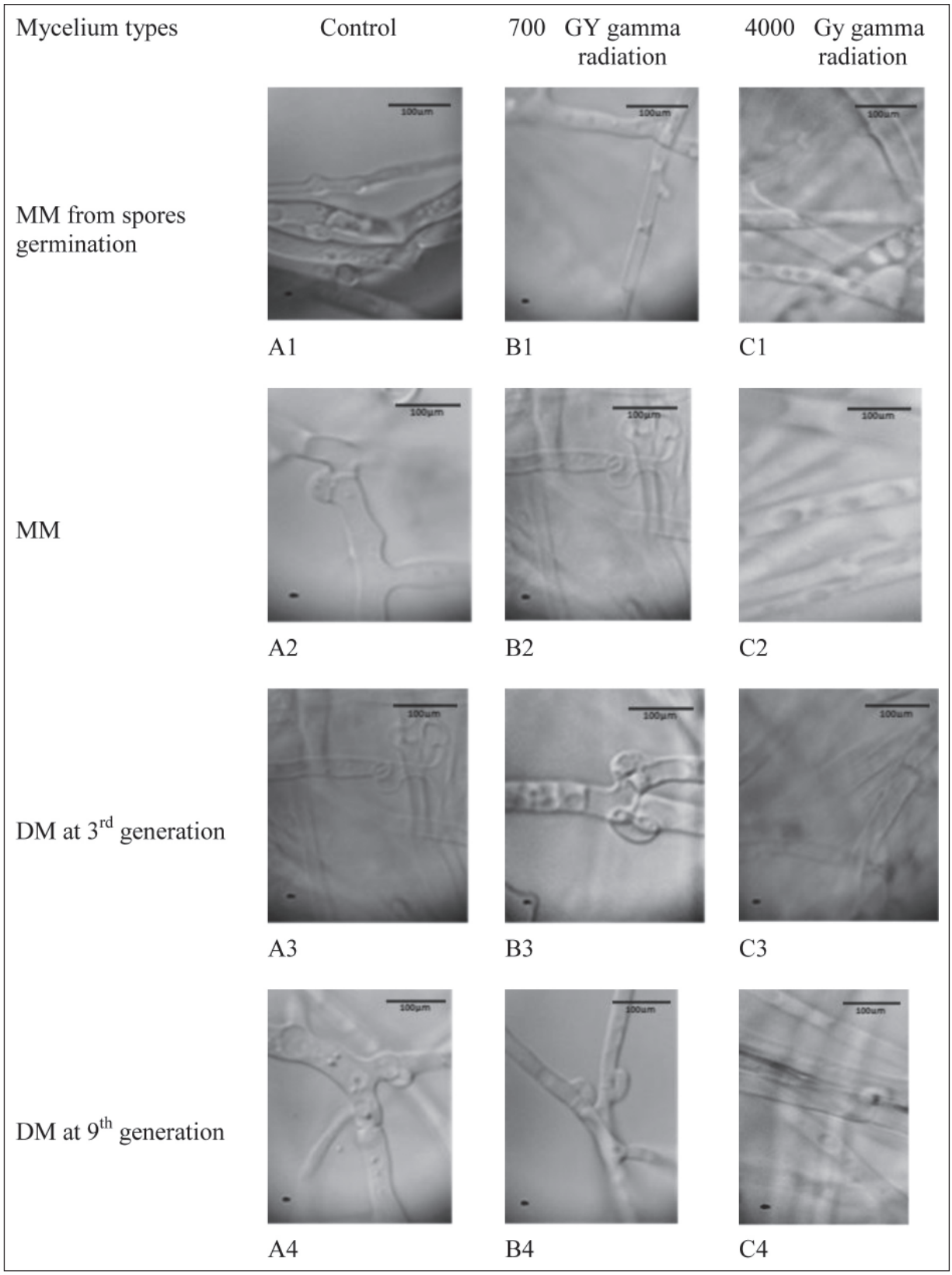

Fig. 5. γ radiation affects the clamps formation. Clamps observed on slides under 40× objective magnification at three different doses. A the control which show the highest counting for the DM, B was the 700 Gy samples and C 4000 Gy samples.

formed are linked to the dose strength, i.e. a higher dose will result in lower number of clamps. This studies focus on the performance of mycelium growth after irradiation, however limited studies have been done towards the formation of fruit bodies from these mycelium. It will be interesting to observe the morphology of the fruit body formed by these mycelium compared to the control, and to investigate the correlation of the phenotype and genotype of this new shiitake variants.

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