

Gamma Irradiation Effect on Embryogenic Callus Growth of *Citrus reticulata* cv. Limau Madu

(Kesan Sinaran Gamma ke atas Pertumbuhan Kalus Embriogenik *Citrus reticulata* cv. Limau Madu)

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ABSTRACT

Induced mutagenesis using gamma ray has been proven applicable to improve varieties of many genotypes of crop species. The effects of ^{60}Co gamma ray dosage on growth and callus induction of nucellus segments of *Citrus reticulata* cv. limau madu were investigated. The nucelli were exposed to gamma rays at doses of 10, 20, 40, 60, 80, 100 and 120 Gy, followed by embryogenic callus (EC) induction on Murashige and Skoog medium supplemented with 500 mg L⁻¹ malt extract (ME), 146 mM sucrose, 0.8% (w/v) agar and 13.3 μM benzyl amino purine (BAP). Survival, callus type and colour, degree of callus formation, time of callus formation and total fresh weight of callus varied among the treatments. All untreated explants (controls) survived and produced friable EC in the 2nd or 3rd week of culture, whereas the irradiated nucelli showed delayed response. EC derived from the nucelli irradiated at 10, 20 and 40 Gy appeared in the 3rd week of culture, whereas EC from the 60 and 80 Gy doses appeared in the 4th week. Exposure to higher doses (100 and 120 Gy) completely suppressed callus formation. After 35 days of culture, an average of 697 and 660 mg of EC were harvested from the nucelli irradiated at 10 and 20 Gy, respectively, which was higher than those at 40 Gy (441 mg), 60 Gy (436 mg) and 80 Gy (380 mg). EC were initiated and proliferated and subsequently regenerated into plantlets. DNA of plantlets from the 20, 40 and 60 Gy exposure were individually amplified and compared to the control for early detection of mutagenesis using retrotransposon, inter simple sequence repeat and markers related to seedlessness. No variants were observed from the plantlets produced.

Keywords: Embryogenic callus; gamma-rays; LD₅₀; limau madu; nucellus

ABSTRAK

Mutagenesis aruhan menggunakan sinaran gamma dibuktikan berupaya untuk menambah baik pelbagai genotip tanaman. Kesan dos sinar ^{60}Co gamma pada pertumbuhan dan induksi kalus pada segmen nuselus *Citrus reticulata* cv. limau madu dikaji. Nuselus didedahkan kepada sinar gamma pada dos 10, 20, 40, 60, 80, 100 dan 120 Gy, diikuti dengan aruhan kalus embriogenik (EC) di atas medium Murashige dan Skoog dengan penambahan 500 mg L⁻¹ ekstrak malt (ME), 146 mM sukrosa, 0.8% (w/v) agar dan 13.3 μM benzil amino purin (BAP). Kemandirian, jenis dan warna kalus, tahap pembentukan kalus; masa pembentukan kalus dan berat segar kalus berbeza antara rawatan. Semua eksplan tidak dirawat (kawalan) hidup dan menghasilkan EC rapuh dalam masa 2 hingga 3 minggu kultur, manakala nuselus radiasi menunjukkan tindak balas yang lambat. EC daripada nuselus diradiasi pada 10, 20 dan 40 Gy muncul pada minggu ke-3 kultur, manakala EC daripada dos Gy 60 dan 80 muncul pada minggu ke-4. Pendedahan kepada dos lebih tinggi (100 dan 120 Gy) telah menghalang pembentukan kalus sepenuhnya. Selepas 35 hari dikultur, purata 697 dan 660 mg EC dicatatkan daripada nuselus yang diradiasi masing-masing pada 10 dan 20 Gy, iaitu lebih tinggi daripada 40 Gy (441 mg), 60 Gy (436 mg) dan 80 Gy (380 mg). EC dimulakan, berkembang biak dan seterusnya dijana semula menjadi anak pokok. DNA anak pokok daripada pendedahan 20, 40 dan 60 Gy telah diamplifikasi secara individu dan dibandingkan dengan kawalan untuk pengesanan awal mutagenesis menggunakan retrotransposon, antara ulangan jujukan ringkas dan penanda berkaitan ketidakhadiran biji benih. Tiada variasi diperhatikan daripada anak pokok yang dihasilkan.

Kata kunci: Kalus embriogenik; LD₅₀; limau madu; nuselus; sinar gamma

INTRODUCTION

Citrus reticulata cv. limau madu is a loose-peel mandarin with spherical-shape fruits and shiny green or greenish yellow peel (Eley et al. 2012; Makeen et al. 2007). The fruit has sweet juice; unattractive fruit colour in mature stages, is seedy and has all of the characteristics of a mandarin. It is susceptible to *Tristeza* virus and *Phytophthora* collar rot

diseases which are considered as the prime threats to the survival of citrus trees (Ko 1997).

The quality of limau madu in Malaysia can be improved through breeding for the desired traits. However, conventional breeding in citrus is slow and difficult because of its complex reproductive biology; such as high heterozygosity, apomixis, polyembryony, cross- and self-

incompatibility, quantitative and qualitative traits in nature while long juvenility are expensive, time-consuming and limited rate of improvement (Gulsen et al. 2010; Xiao et al. 2009). *Citrus* improvement for many years therefore has been mainly based on the selection of naturally occurring somatic mutants (Xiao et al. 2009).

Induced mutagenesis of crops has been successfully utilized to increase frequencies of somatic variants. Mutagenesis offers the possibility of changing only one or a few traits of first-rate cultivars, which can further enhance quality and quantity of crops (Xu et al. 2012) while preserving the overall traits. Maluszynski et al. (1995) stated that induced mutations (*in vivo* and *in vitro*) have been employed primarily to improve particular traits in well-adapted local varieties or to generate variations difficult to find in germplasm collections. There has been success with the application of induced mutation for improvement of some vegetatively propagated crops and banana has reportedly benefited more than any other fruit crop from this technology (Predieri 2001). Induced mutagenesis using gamma ray has been proven applicable to improve characteristics of many citrus genotypes such as seedlessness (Chen et al. 1991; Froneman et al. 1996; Hearn 1986; Micke et al. 1985; Spiegel-Roy et al. 1990, 1985; Sutarto et al. 2009) and spinelessness (Kukimura et al. 1976) and for inducing changes in fruit and juice colour (Sutarto et al. 2009; Wu et al. 1986).

Recent mutant development and selection have been frequently carried out under tissue culture protocols due to their simplicity, efficiency and reliance on relatively inexpensive tools (Ahloowalia 1998). This combination of induced mutagenesis and tissue culture offers several advantages over conventional methods by, for example, increasing mutation induction efficiency and uniformity, allowing the handling of large populations of mutated cells of minute size, providing the possibility of mutant recovery and speedy production of selected variants and facilitating *in vitro* selection (Predieri & di Virgilio 2007; van Harten 1998).

Rapid development in molecular biotechnology has also partially solved some of the limitations associated with conventional citrus breeding (Xiao et al. 2009). Early genetic detection of variants developed *in vitro* for crop improvement confirms the success of breeding programmes such as that reported for *Ochreinauclea missionis* (Chandrika & Rai 2009), Greek fir (Krajnáková et al. 2011) and *Cymbopogon pendulus* (Bhattacharya et al. 2010). Markers used for this purpose include intersimple sequence repeat (ISSR) primers, extensively useful in establishing the genetic stability of *in vitro*-regenerated plantlets in many crop species (Joshi & Dhawan 2007; Lakshmanan et al. 2007) and retrotransposons, which contain long, defined, conserved sequences that can be used for cloning of specific markers and flanking sequences (Kalendar et al. 1999). The effects of gamma irradiation on *Citrus reticulata* cv. limau madu nucellus culture is reported in this paper. Plantlets derived from culture were tested for early indication of genetic variations.

MATERIALS AND METHODS

PLANT MATERIAL AND SAMPLE PREPARATION

Open-pollinated, immature fruits of *Citrus reticulata* cv. limau madu at different ages (80 - 120 days after anthesis) were harvested from mature plants at Desaru Fruit Farm, Desaru, Johor Bahru, Malaysia. Handling and sterilization of fruits were as described by Agisimanto et al. (2012). Under aseptic conditions in a laminar air flow cabinet immature seeds along the segment wall adjacent to fruit axis were carefully excised and the interguments (testa and tegment) were then removed with the aid of a dissecting microscope. The nucelli were cultured on plant growth regulator (PGR)-free Murashige and Skoog (MS) (1962) medium.

GAMMA RAY RADIATION TREATMENT

A certified Gamma Cell (model 220) was used as the source of ^{60}Co for inducing mutagenesis. The unit for the absorbed dose of radiation energy is the gray (Gy), which is equivalent to 1 J Kg^{-1} and 100 rads. Each dose (10, 20, 40, 60, 80 and 100 Gy) was administered to 217 healthy nucelli planted on plant growth regulator (PGR)-free medium a week after culture initiation. After irradiation, the nucelli were directly transferred to the culture medium to induce embryogenic callus initiation.

CULTURE MEDIUM AND CONDITIONS

MS basal salt and vitamin (MS) (Duchefa M220) medium was used as the basic medium and was supplemented with 500 mg L^{-1} malt extract (ME), 146 mM sucrose, 0.8% (w/v) agar and $13.3 \text{ }\mu\text{M}$ benzyl amino purine (BAP) for callus initiation and proliferation. The pH was adjusted to 5.7 ± 0.1 with 0.5 M potassium hydroxide before autoclaving at 121°C and 15 psi (103 kPa) for 15 min. Petri dishes ($90 \times 15 \text{ mm}$) were filled with 25 mL of the medium and sealed with parafilm prior to use. The cultures were maintained in a growth room at a temperature of $23 \pm 2^\circ\text{C}$ with a 16 h photoperiod under cool-white light at $60 \text{ }\mu\text{mol m}^{-2} \text{ s}^{-1}$ provided by Osram cool-white fluorescent lamps. The embryogenic calli (EC) were subcultured biweekly on to the fresh medium. EC suspension culture was initiated and proliferated following Agisimanto et al. (2012). For somatic embryo development, MS with 110 mM sorbitol, 36 mM galactose, 500 mg/L ME were used and for plantlet development, MS with 146 mM sucrose, 0.27 gelrite were employed (Agisimanto et al. 2012).

GENETIC VARIATIONS OF PLANTLETS DERIVED FROM IRRADIATED NUCELLI

Total DNA was extracted from the samples following the cetyl trimethylammonium bromide (CTAB) protocol described by Doyle and Doyle (1990) with slight modification. Approximately 100 mg of leaf of plantlet was ground using $400 \text{ }\mu\text{L}$ of preheated (65°C) extraction

buffer (2% CTAB, 20 mM EDTA, 100 mM Tris-HCl, 1.4 M NaCl, 2% polyvinylpyrrolidone (PVP), 0.2% mercaptoethanol), transferred to a centrifuge tube (2 mL) and incubated for 30 min in a 65°C water bath, inverted every 10 min. Subsequently, approximately 1 mL of chloroform-isoamyl alcohol (24:1) was carefully mixed and the cells were centrifuged at 12000 rpm for 15 min at room temperature. The supernatant was collected and carefully mixed with 0.1 volumes of 3 M sodium acetate and ice-cold absolute ethanol. The DNA samples were collected by high-speed centrifugation for 10 s, and then carefully washed with ice-cold absolute and 70% ethanol, respectively. Finally, the samples were dried at room temperature and dissolved in 50-100 µL of TE (10 mM Tris and 0.1 mM EDTA). The quality and amount of DNA were determined by spectrophotometry and agarose-gel analysis with lambda DNA, as described by Sambrook et al. (1989). For DNA amplification, three ISSR primers, namely, (GA)₈YG, (TCC)₅RY and HVH(CA)₈, from previous genetic analysis of citrus (Fang & Roose 1997; Fang et al. 1997) were used. PCR was performed as follows: 94°C for 3 min; followed by 28 cycles of 94°C for 45 min, 53°C for 1 min and 72°C for 2 min and the final extension step at 72°C for 10 min. The PCR component comprised 20 µL of 100-ng genomic DNA, 200 mM dNTP, 3 mM MgCl₂ in buffer (Promega), 250 mM primer and 1 U of Taq DNA polymerase (Promega). PCR for parthenocarpic marker (seedlessness related marker) DefH9 was performed in a 25 µL-reaction mixture containing 20 ng of DNA, 1X PCR buffer with MgCl₂, 5 µM of each primer, 2 µL dNTPs of 200 nM and 1U of Taq DNA polymerase (Promega). PCR was performed as follows: 94°C for 5 min; followed by 29 cycles of 94°C for 1 min, 49°C for 1 min and 72°C for 2 min and final extension step at 72°C for 10 min. PCR for retrotransposon marker LTR was performed with 100 ng of genomic DNA, 200 mM dNTPs, 1.5 mM MgCl₂, 200 nM of each degenerated primer and 1 U of Taq DNA polymerase. PCR was performed as follows: 94°C for 5 min; followed by 30 cycles of 94°C for 1 min, 45°C for 1 min and 72°C for 2 min and final extension step at 72°C for 10 min. The PCR products were separated using

1.8% agarose gel in 1× Tris-borate-EDTA (TBE) buffer for 1 h at 100 V.

EXPERIMENTAL DETAILS AND STATISTICAL ANALYSIS

The irradiation experiment was performed in a completely randomised design with 3 replications. Percentage of survival, type and colour of callus, degree of callus formation and time of callus formation were recorded every week and the total callus fresh weight was recorded at 35 days after irradiation prior to the next subculture. Data were analyzed by analysis of variance and mean comparison was made using Duncan Multiple Range Test (DMRT) using SAS and regression analysis with Microsoft Excel software. Probabilities of less than or equal to 0.05 were considered significant. Values in all tables represent the mean ± SD of samples.

RESULTS

SURVIVAL OF EXPLANTS AND CALLUS INITIATION

Nucelli were cultured a week before irradiation to ensure the nucelli at an actively-growing stage for the irradiation treatments. In the initial cultures, the explants were light-yellowish in appearance, but after the gamma irradiation treatments, the tissue of nucelli tended to be dark-yellowish. Embryogenic calli were formed mostly around the micropylar end (Figure 1 & 2). Surviving explants were indicated by callus formation on the nucelli by the 2nd to 4th week after treatment. Callus formation was clearly affected by the different doses of gamma rays (Table 1).

The control survived 100%. The percentage of survival reduced with increasing doses of irradiation. Only 7.41% callus survived from treatment using 80 Gy. At lower dose of radiation (0-20 Gy), the nucelli effectively produced friable embryogenic calli (FEC). At all irradiation doses, except at 80 Gy, the morphology and performance of the primary calli were generally white and creamy and exhibited faster growth in forming FEC tissue. The nucelli

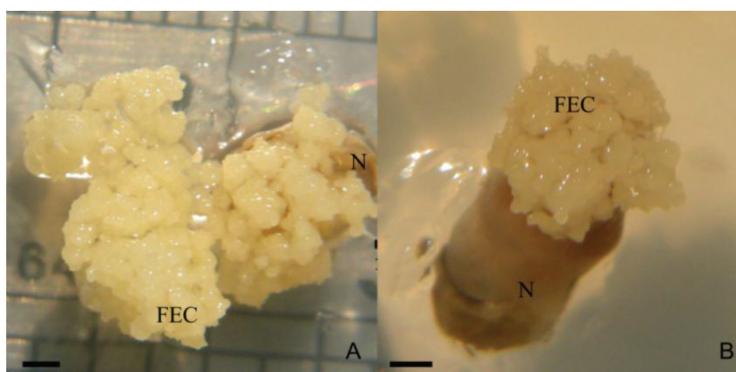


FIGURE 1. Embryogenic callus from irradiated nucellus of *Citrus reticulata* cv. limau madu on MS semisolid culture. Nucellus responses (N) of control (a) and irradiated (b) at day 35 after irradiation on medium with BAP at 3 mg L⁻¹ and sucrose at 146 mM. (Bar = 1 mm)

irradiated at 80 Gy produced compact calli and thereafter embryogenic calli by the 4th week of culture.

Based on time interval to produce callus, the survival percentages can be classified into three groups (Table 1). The first group consists of control, which started forming calli in the 2nd week after initiation; the second and third groups comprise of nucelli treated with 10-40 and 60-80

Gy, respectively. These groups started forming callus in the 3rd and 4th weeks after irradiation, respectively. Hence, compared with control, the irradiated nucelli were delayed in forming callus and varied in their capacity to produce primary callus. The nucelli treated with 100 and 120 Gy were completely brown at the 2nd week and remained inactive throughout the 35 days of culture (Figure 2).

TABLE 1. Effects of gamma irradiation doses on nucellus performance of *Citrus reticulata* cv. limau madu at day 35 of culture

Dose (Gy)	SP (%)	CT	CC	DCF	TCF (weeks)	TFW (mg)
0	100.0 ^a	FEC/ compact	white/ green	+++++	2	678.0±10.4 ^a
10	50.00 ^a	FEC	White	+++++	3	697.67±19.6 ^a
20	45.45 ^a	FEC	White	+++++	3	660.58±18.8 ^a
40	21.88 ^{ab}	FEC	White	+++	3	441.44±23.8 ^b
60	13.33 ^b	FEC	White	++	4	436.67±24.8 ^b
80	7.41 ^b	FEC/ compact	White	+	4	380.42±7.6 ^c
100	0	-	-	-	-	-
120	0	-	-	-	-	-

Means followed by the same letter are not significantly different at $p \leq 0.05$ according to the DMRT. The survival percentages (SP), type of callus (CT), colour of the callus (CC), degree of callus formation (DCF), time to callus formation (TCF), total fresh weight (TFW), Friable embryogenic callus (FEC). +: very weak; ++: weak; +++: moderate; ++++: profuse; +++++: very profuse

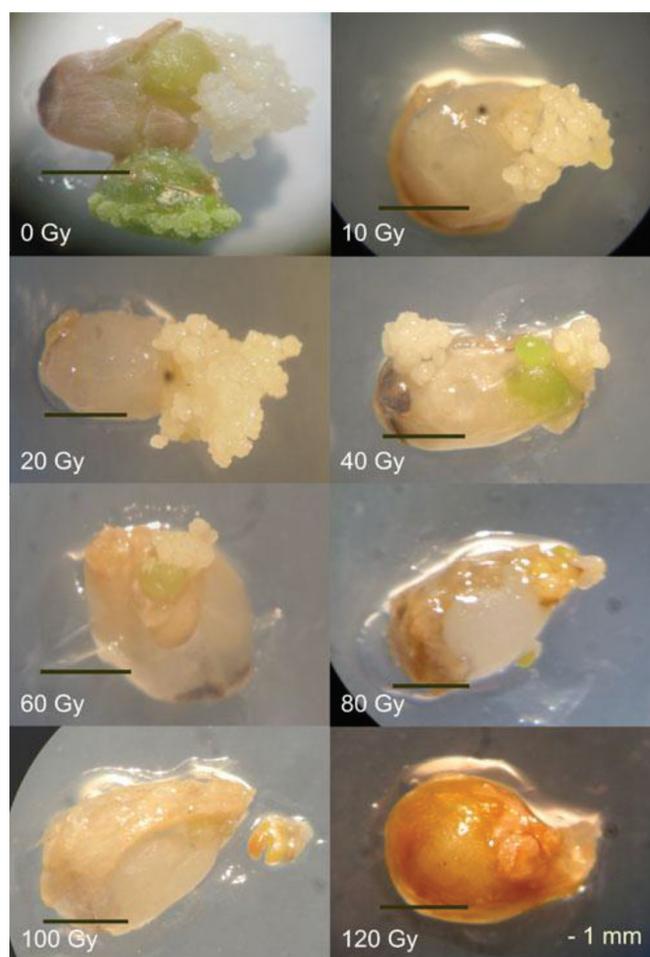


FIGURE 2. Effect of gamma doses on nucelli of *Citrus reticulata* cv. limau madu at day 35 after initiation. Bar = 1 mm

During the early period of callus formation, response of explants to the different treatments was initially variable, indicating various responses to the irradiation. At the lower doses of gamma rays it was found that radiation at 10 and 20 Gy had similar callus induction as the control as shown in the total fresh weight (Table 1). However, further increase of radiation (40, 60 and 80 Gy) suppressed the FEC growth and significantly lowered the fresh weights. Nevertheless, after the first sub-culture, all callus formed on the irradiated explants grew normally regardless of the treatment (Figure 3).

RADIO-SENSITIVITY TEST OF NUCELLUS EXPLANTS

Survival percentage of the nucelli in relation to the level of irradiation dose (within 35 days) are summarised in Figure 4. The findings indicate that as the irradiation dose increased, higher frequency of nucellus growth reduction was observed. In fact, gamma radiation at 10 Gy suppressed 50% of the active nucelli. Radiation at 20, 40, 60 and 80 Gy suppressed growth by 54.6, 78.1, 86.7 and 92.6%, respectively. Exposure to higher doses of 100 and 120 Gy completely suppressed and killed the nucelli. Thus these doses of radiation (100 and 120 Gy) are categorised

as lethal doses for the nucelli of *Citrus reticulata* cv. limau madu. There were significant differences ($p \leq 0.05$) between controls and the treated explants, especially among doses between 20 and 60 Gy. After regression analysis ($R^2 = 0.90$), the estimated lethal dose 50% (LD_{50}) was determined to be 30 Gy.

GENETIC VARIATIONS OF PLANTLETS DERIVED FROM IRRADIATED NUCELLI

The DNA of ten plantlets regenerated from cells isolated from nucelli irradiated individually using 20, 40 and 60 Gy were isolated and amplified. The banding patterns of the ten plantlets are shown in Figure 5. Primers which were generated from repetitive sequences namely retrotransposon marker (LTR); microsatellite $(GA)_8YG$, $(TCC)_5RY$, $HVH(CA)_8$ and parthenocarpic marker (DefH9) amplified genome samples. However, none of them showed variation compared with control.

DISCUSSION

Availability of efficient *in vitro* plant-regenerating systems has extended the potential use of *in vitro*

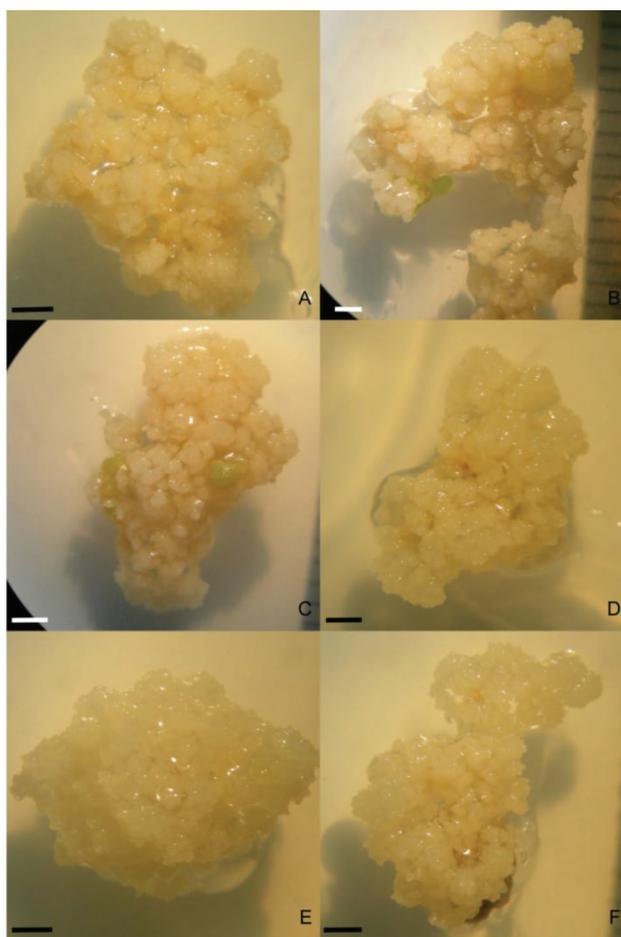


FIGURE 3. Callus derived gamma irradiation of *Citrus reticulata* cv. limau madu after 4 biweekly subculture. A (0 Gy), B (10 Gy), C (20 Gy), D (40 Gy), E (60 Gy), F (80 Gy). Bar = 1 mm

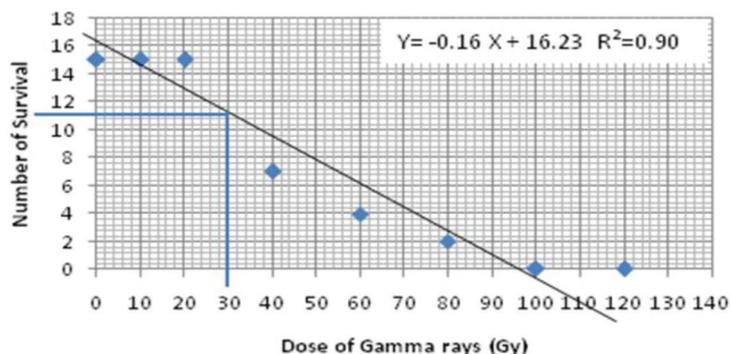


FIGURE 4. Radio-sensitivity curve of *Citrus reticulata* cv. limau madu at day 35 after initiation

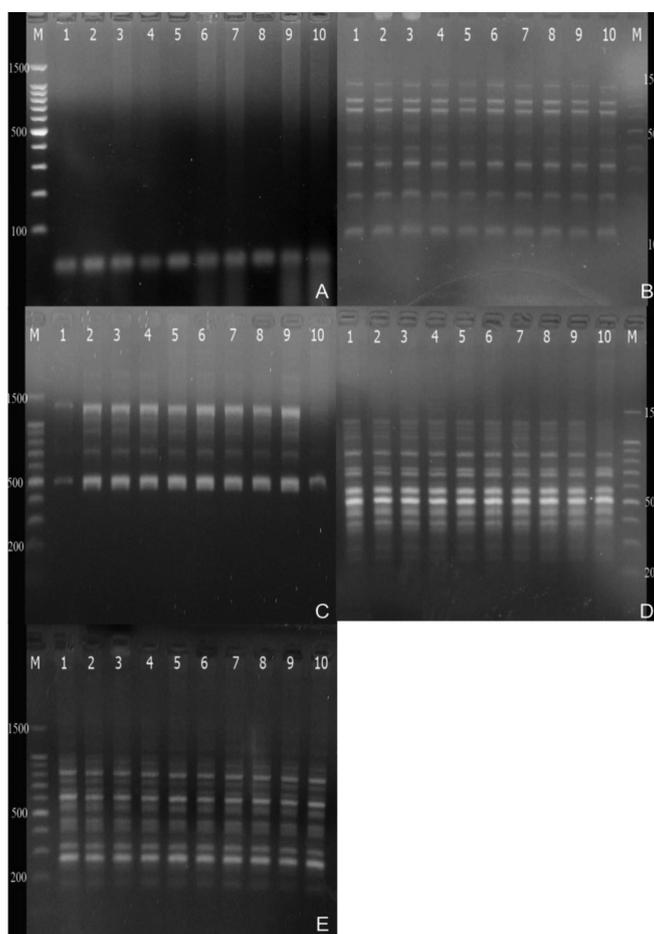


FIGURE 5. PCR-based screening for detecting variation of *Citrus reticulata* cv. limau madu irradiated with gamma rays. Primer DefH9 (A), LTR (B), ISSR primer $(TCC)_3RY$ (C), $HVH(CA)_8$ (D) and $(GA)_8YG$ (E) were used to amplify plantlet from explants irradiated by 0 Gy (line 1), 20 Gy (line 2-8), 40 Gy (line 9) and 60 Gy (line 10). M was 100 bp

culture-induced mutation (*in vitro* mutagenesis) in development of superior varieties for crop improvement (Patade & Suprasanna 2008). Gamma rays are frequently employed for induced mutation due to its low energy and stabilization with a high and uniform penetration in plant tissues (Jain 2002). In this study, the frequencies of callus formed and time to form callus were significantly

affected by level of irradiation dose. Callus initiation and growth reduced linearly with increasing doses. At lower doses, friable embryogenic callus initiation was delayed 1-2 weeks beyond the time required for untreated nucelli and developed more profusely, whereas higher levels of irradiation resulted in increasing suppression of cell growth. Survival of nucelli decreased with increasing

dose and nucelli were apparently dead, at the highest doses (100-120 Gy). The difference in the response in callus formation was considered as the first indication of cellular changes in the irradiated explants, reflecting differences in the ability of nucelli to survive to specific radiation-induced cellular damage and to continue their growth thereafter.

It is generally agreed that lower doses of gamma rays stimulate cell division, growth and development in various organisms, both animal and plant. The amount of callus produced by nucelli irradiated at 10 Gy at the 5th week of culture was higher though not significant than the control. Stimulation response of tissue culture in term of callus induction, embryogenesis or regeneration response at lower doses of irradiation has previously been demonstrated for example in banana, grapevine, rice and *Etilingera elatior* (Charbaji & Nabulsi 1999; Chen et al. 2001; Kulkarni et al. 2004, 1997; Yunus et al. 2013). Witjaksono and Litz (2004) successfully induced embryogenic culture of avocado zygotic embryos using gamma rays at 0 to 50 Gy and a lower dose of 10 Gy was found to increase cell growth and embryo formation. The effects of higher doses of gamma irradiation however, might result from its disruption of cells, which may lead to mutation and/or the creation of more variants. The biological effect of gamma rays is based on interaction between ionizing gamma with atoms or molecules in the cell, particularly water. They have enough energy to damage chemical bounds, causing a photochemical reaction. Free hydroxyl and other reactive radicals are released and able to diffuse and damage important molecules in plant cell and DNA as well. Plant cells seem to respond to high accumulations of energy by losing vitality and the capacity to adjust themselves to the new physiological changes, ending in death of the cell and organism (Kleffel et al. 1986).

The mean lethal irradiation dose of 50% (LD_{50}) was used to determine radiosensitivity of *Citrus reticulata* cv. limau madu nucelli to gamma rays. LD_{50} , determined by regression, was found to be at 30 Gy. Even though the LD_{50} is generally used in many crops as an optimal dose for mutation induction, in the present study, the range of 20 to 40 Gy was associated with a drastic decrease in callus induction/survival. Thus, the optimal radiation dose for mutation induction for *Citrus reticulata* cv. limau madu is approximated at 20 Gy, which is associated with a decrease of more than 50% in survival and callus induction. A number of available reports suggest that *in vitro* tissue and callus were more sensitive to radiation treatment and required much lower doses than seed or other plant organs (Ahloowalia & Maluszynski 2001; Chen et al. 2001; Misra et al. 2003).

Irradiation tended to suppress cell growth and development; however, when irradiated cultures proliferated in suspension cell culture, the negative effect of irradiation on somatic embryo development was eliminated. Witjaksono and Litz (2004) suggested

that limited number of proliferation cycles of irradiated embryogenic cultures is essential in order to assure recovery of sufficient numbers of somatic embryos for subsequent development and germination. The use of cell culture for proliferation also holds great promise since the incidence of chimera would be minimal (Mba 2013).

In the present study plantlets used for genetic analysis were generated sequentially from the first generation of cells induced from gamma irradiation. There was no variation observed. According to Toker et al. (2007) mutations are mostly recessive and the desired traits may not be selected for until the second generation (M_2) of the plants derived from irradiated cells. However, unlike recessive mutations, dominant mutations occur at low frequencies and they can be selected for in the first (M_1) generation (Micke & Donini 1993). Therefore, dominant mutation induced by gamma-rays may not have occurred in the present study and the recessive ones have yet to be observed in the subsequent generations.

CONCLUSION

Callus initiation and friable embryogenic callus formation of *Citrus reticulata* cv. limau madu from nucellus explants were significantly affected by the level of doses of gamma rays. The effective doses of gamma irradiation for inducing 50% growth reduction of nucelli were in the range of 20-40 Gy. The lethal dose of 50% (LD_{50}), as determined by regression analysis, was 30 Gy. At lower doses of radiation (10-20 Gy), the nucelli gave higher FEC induction at a faster rate than those irradiated at higher doses. There was no early indication of genetic variation observed in the plantlets derived from the irradiated nucelli.

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