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In vitro regeneration and induction of mutation in Loquat (*Eriobotrya japonica* L.)

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Abstract

Loquat (*Eriobotrya japonica* L.) is a potential fruit crop in Indonesia. The experiments were carried out to study the effect of gamma rays as *in vitro* mutagenesis agent and also the regeneration of mutant explants of loquat on different media. The experiment used two media (MS and WPM) containing BA and NAA, three type of explants (apical shoot, stem, cotyledon), and four doses of gamma rays (0, 10, 30, 50 Gy). The results showed that MS media was better than WPM media in term of callus production. Moreover, explants of loquat showed a different response to gamma irradiation and the highest response on number of shoots, number of leaves and plant height was found at a dose of 10 Gy. On the other hand, the explants of cotyledon were only able to produce callus. The explants of the apical shoot were able to produce the highest number of leaves and shoots than the explants of the stem. However, the combination of MS media, apical shoot explants and 10 Gy of gamma rays irradiation showed a better response in the growth traits of callus diameter, callus height, number of shoots, number of leaves and plantlet height. Overall, the results of this study showed that *in vitro* culture in combination with induced mutations can speed up breeding programmes.

Keywords: Loquat; *in vitro*; mutation; gamma rays.
Abbreviations: MS_Murashige and Skoog; WPM_Woody Plant Medium; BA_Benzyl Adenin; NAA_ Naphthalene Acetic Acid; Gy_Gray

Introduction

Loquat (*Eriobotrya japonica* L.), one of the collection in Cibodas Botanical Garden, is an important evergreen fruit tree of tropical regions that originated from China (Lin et al., 2004). As an important fruit tree, it has high economic potential. Presently, it is mainly cultivated in China, Spain, Turkey, Pakistan, India, Italy and Brazil. It has been grown in more than thirty countries such as Japan, Greece, Israel, Reunion Island, Mauritius Island, USA, Australia, Madagascar, New Zealand and South Africa (Badenes et al., 2000; Vilanova et al., 2001; Feng et al., 2007). In Indonesia, Silalahi et al. (2011) reported that it is grown in several places such as North Sumatra (mainly Karo, Tapanuli Utara, Simalungun, Toba Samosir, and Dairi), West Java (Cipanas), and North Celebes (Tondano). Moreover, the loquat cultivation is still traditionally practiced by farmers, so its production is still very low and could not fulfill consumers demand. However, until recently it has been almost neglected in term of research in Indonesia. Most of the loquat orchards were established using seeds, grafting and shoots cutting due to the unavailability of certified seedling and less information about the technology of cultivation.

Furthermore, the cultivation of a good cultivar may increase the production of loquat. In order to create desired genotypes and phenotypes of loquat we require optimization of biotechnological techniques in this species. The induced mutation has become an established tool in plant breeding to supplement existing germplasm and to improve cultivars in certain specific traits. Predieri (2001) reported that induced mutations change only one or a few specific traits of elite cultivars. It can contribute to fruit improvement without upsetting neither the requirements of the fruit industry nor the consumers. Induced mutations can be created either by specific treatments with physical or chemical mutagens. Moreover, Blasco et al. (2016) reported that gamma-ray irradiation has been successful to obtain parthenogenetic haploids in loquat. *In vitro* propagation is another important technique for the production of seedling, which guarantees quality and safety compared to traditional production. Biotechnological tools for *in vitro* propagation of different tree species have been introduced recently (Drew, 2011; Pijut et al., 2012), and micropropagation protocols are available for many species and cultivars (Ruzic and Vujovic, 2008). However, propagation of woody plants *in vitro* is an intricate process (Abbasi et al., 2013). Clonal propagation of loquat using *in vitro* techniques has been carried out earlier (Lomtatidze et al., 2009), but there is a need to optimize specific protocols as no trials have been made for *in vitro* regeneration of loquat using irradiation with gamma rays. The objective of this experiment was to study the effect of gamma rays and *in vitro* regeneration of loquat.

Results

Based on five parameters, MS media showed better response than WPM media (Fig. 1). Although MS media had a better response, the significant differences were only obtained on callus diameter and height. Moreover, the parameters of plant height, number of shoots, and number of leaves are not affected by MS and WPM media. Sokolov et al. (2014) reported that the combinations of Murashige and Skoog (MS) basal salts with 6-benzylaminopurine and α-naphthaleneacetic acid shown a higher number of shoots in *Magnolia* than the combinations of Woody Plant Media (WPM) with 6-benzylaminopurine and α-naphthaleneacetic acid. On the other hands, the direct regeneration and the induction of a number of multiple shoots/explants depends on the concentration and combination of plant growth regulators present in the media (Ebrahimzadeh et al., 2016). Table 1 shows the differences compositions of MS and WPM basal media.

The highest value of callus diameter and height was obtained using non-irradiated (0 Gy) sample of loquat. Callus diameter and height value of irradiated loquat decreased significantly compared to non-irradiated loquat (Fig. 2). The sample of loquat irradiated with 10 Gy significantly increased number of shoots, number of leaves, and plantlet height compared to 0, 30 and 50 Gy. Moreover, Hasbullah et al. (2012) reported that growth responses of callus were strongly influenced by the radiation dose. We also observed that the callus diameter and callus height were decreased compared to the control.

Three types of explants of loquat showed differences response to the media. The explants from apical shoot gave the highest value for the number of shoots (1.79) and leaves (5.42). On the other hands, the explants of cotyledon showed only produced callus (Fig. 3). The plant regeneration system of cotyledonous explants is one of the best *in vitro* regeneration systems, which could yield lots of sterile explants in a short time (He et al., 2007). Moreover, Nayak and Kalidass (2016) reported lack of morphological changes due to organogenesis from cotyledon explants. The cotyledons cultured in MS basal medium failed to show any morphological changes regardless of growth regulators. Addition of cytokinin like BAP had a positive effect on shoot formation from the cotyledon explants.

The interaction between three combination factors gave significantly different response on five parameters of *in vitro* regeneration of loquat after subculture (Table 2). Although not statistically different, the combination of WPM and apical shoot showed highest number of shoots and leaves than MS and apical shoot combination. The opposite results were observed in plant height, in which the apical shoot combination was higher in MS (1.1083 cm) compared to WPM (1.0917 cm). The interaction between media and gamma radiation combination showed that the combination of MS and 10 Gy had higher value on the number of shoots, number of leaves and plant height compared to other combination between media and gamma radiation. On the interaction between explants and gamma radiation, the results showed that the explants of apical shoot irradiated by 10 Gy of gamma rays had a better effect on number of shoots, number of leaves and plant height than others combination. Furthermore, the interaction between three combination factors showed that the combination of MS + apical shoot + 10 Gy has the best effect on number of shoots (3.67), number of leaves (10.00) and plant height (2.20 cm). On the other hands, the highest callus diameter and callus height were shown by the combination of MS + apical shoot + 0 Gy (1.60 cm) and MS + stem + 0 Gy (1.20 cm).

Discussion

The explants type, planting media and irradiation doses of gamma rays affected *in vitro* propagation of loquat. The *in vitro* propagation was conducted on shoots, stem, and cotyledons of germinating seeds or seedlings developed under lab condition. According to our results, each type of explants gave a different response to planting media and doses of gamma rays. At *in vitro* level, the interaction between explants, planting media and doses of gamma rays were also evident.

In this research, the apical shoot explants showed the best growth compared to stem and cotyledon. All other parameters such as number of shoots, number of leaves, plant height, also diameter and height of callus were higher when in vitro culture was carried out by apical shoot. Meanwhile, the cotyledons only created callus (Fig. 3). The factor of genotype and source of explant influenced growth and morphogenesis of tissue culture. Each cell of plant has different characteristics. The young and meristematic part of the plant is the best explant for explant source (Armini et al., 1991). Shoots have cells with faster splitting ability compared with stem or cotyledon of endosperm, so the organogenesis by shoots explant is better than with other treatments.

Generally, each species requires different planting media and nutrients. Two basal media, Murashige and Skoog (MS) and Woody Plant Medium (WPM) were used during the experiment. Furthermore, MS medium and WPM enriched with BA 2 mg/L + NAA 0.1 mg/L as regeneration medium. Mariska and Ragapadmi (2001) reported that WPM is the best medium for growth and development of woody plants. Moreover, Pardal et al. (2004) also reported that WPM is widely used in various species of woody plants, because it has low total ion content, with high sulfate component. The magnesium is a macro element in WPM that strongly supports plant tissue growth. Therefore, WPM media is expected to produce better growth for loquat culture which is also a woody annual plant. However, the results showed that MS medium has better results than WPM on all growth parameters of plant (Fig. 1). The specialty of MS medium is a high content of nitrate, potassium, and ammonium. In addition, it has a decent amount of inorganic elements for plant tissue culture growth (Wetter and Constabel, 1991).

The appropriate combination of nutrients and plant growth regulators are needed for plantlet formations. The addition of plant growth regulators is expected to stimulate the differentiation of explant. Moreover, the plant growth regulators activities depend on the type, chemical structure, concentration, plant genotype and physiology (Satyavathi et al., 2004). Probably, the addition of BA and NAA as exogenous cytokinin and auxin could increase the cell metabolism and explant growth.

Table 1. Comparison MS and WPM basal media.

Compound	MS	WPM
Macronutrients (mM/L)		
$CaCl_2$	2.99	0.65
KNO_3	18.79	-
K_2SO_4	-	5.68
KH_2PO_4	1.25	1.25
$MgSO_4$	1.50	1.50
NH_4NO_3	20.61	5.00
$Ca(NO_3)_2$ $2H_2O$	-	2.35
Microelements (µM/L)		
$CoCl_2$ $6H_2O$	0.11	-
$CuSO_4$ $5H_2O$	0.10	1.00
H_3BO_3	100	100
KI	5.0	-
$MnSO_4$ H_2O	100.0	130.0
Na_2MoO_4 $2H_2O$	1.03	1.03
$ZnSO_4$ $7H_2O$	29.91	29.91
Fe Na EDTA	100.0	100.0
Vitamins (µM/L)		
Myoinositol	556.0	556.0
Thiamine hydrochloride	0.30	2.96
Nicotine acid	4.10	4.06
Glycine	26.64	26.64
Pyridoxine hydrochloride	2.43	2.43

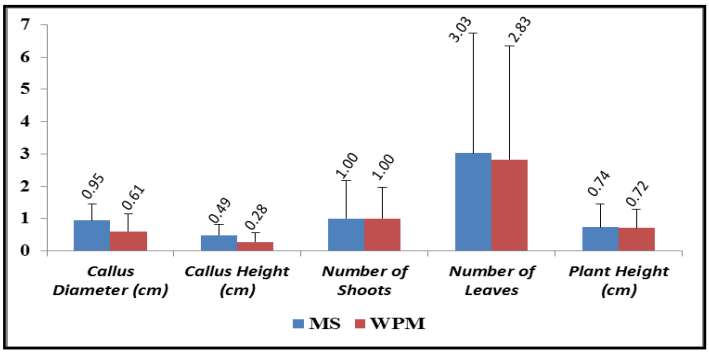

Fig 1. The effect of media on *in vitro* regeneration of loquat, 15 weeks after subcultures.

The use of plant growth regulator in tissue culture depends on the purpose or desired growth. BA (Benzyl Adenine) becomes a commonly used hormone to stimulate shoot multiplication, because it has strong activity compared to kinetin (Zaer and Mapes, l982). BA has the same basic structure as kinetin, but it is more effective for multiplication due to BA has benzyl groups (George and Sherington, l984). Flick et al. (1993) reported that in order to get a good result for *in vitro* shoot production, BA has better effect compared to kinetin and 2-iP. Meanwhile, NAA as a synthetic auxin works in root formation of plantlets. Rapid growth and multiplication of shoots are based on the quantity and quality of cytokinins and auxins in media as well as on their endogenous levels in plants, which vary from species to species and between growth phases (Panjaitan et al., 2007). Our study shows that buds could be formed from the explants of shoots and stems with the combination of BA and NAA. On the other hands, the roots did not form even 15 weeks after planting. Huetteman and Preece (1993) reported that cytokinin releases apical dominance, which hastens axillary bud formation and reduces the length of explants. Moreover, George and Sherrington (1984) reported that the natural cytokines in the explant can stimulate the explant to form buds.

The interaction between MS media, apical shoot explant and 10 Gy of radiation doses can increase the number of shoots, leaves and plant height (Table 2). From the results, 10 Gy is the best radiation doses to increase the growth plantlets (Fig. 2). Meanwhile, the interaction between MS media, apical shoot and stem explant at 0 Gy of radiation dose could increase diameter and height of callus (Table 2). New leaves were produced on the shoots and stem explant. The growth of leaves was affected by the nitrogen content in the media. The leaves are very important for photosynthesis process. The better growth explant can be indicated from the number of leaves. According to Hendaryono and Wijayani (1994), magnesium elements can increase the phosphate content in plants. Phosphate is a raw material for the formation of proteins and formation of leaves.

Table 2. The interaction between media, explants and gamma radiation on *in vitro* regeneration of loquat (*Eriobotrya japonica*), 15 weeks after subculturing.

Interactions	Callus Diameter (cm)	Callus Height (cm)	Number of Shoots	Number of Leaves	Plant Height (cm)
Media x Explants					
MS + apical shoot	0.9625 cd	0.3875 a	1.7500 c	5.1667 c	1.1083 b
MS + stem	0.7500 b	0.5333 b	1.2500 b	3.9167 bc	1.1083 b
MS + cotyledon	1.1375 d	0.5500 b	0.0000 a	0.0000 a	0.0000 a
WPM + apical shoot	0.5250 a	0.2917 a	1.8333 c	5.6667 c	1.0917 b
WPM + stem	0.7917 bc	0.2667 a	1.1667 b	2.8333 b	1.0583 b
WPM + cotyledon	0.5083 a	0.2917 a	0.0000 a	0.0000 a	0.0000 a
Media x Gamma Radiation					
MS + 0 Gy	1.4000 e	0.7667 d	1.3333 cd	3.1667 b	0.7667 bc
MS + 10 Gy	1.0889 d	0.5056 c	1.8889 e	6.1667 c	1.1222 d
MS + 30 Gy	0.9111 cd	0.4889 c	0.7778 b	2.4444 ab	0.6333 ab
MS + 50 Gy	0.4000 b	0.2000 b	0.0000 a	0.3333 a	0.4333 a
WPM + 0 Gy	1.0880 d	0.4889 c	1.0000 bc	3.4444 b	0.9000 c
WPM + 10 Gy	0.8556 c	0.4222 c	1.4444 d	3.6667 b	0.6889 bc
WPM + 30 Gy	0.4889 b	0.2222 b	0.8889 b	2.8889 b	0.6778 bc
WPM + 50 Gy	0.0000 a	0.0000 a	0.6667 b	1.3333 ab	0.6000 ab
Explants x Gamma Radiation					
Apical shoot + 0 Gy	1.4167 f	0.5667 ef	2.0000 d	6.6667 d	1.4500 e
Apical shoot + 10 Gy	0.9583 de	0.4583 cde	3.1667 e	8.3333 d	1.6833 e
Apical shoot + 30 Gy	0.6000 bc	0.3333 bcd	1.5000 cd	5.6667 cd	0.8333 c
Apical shoot + 50 Gy	0.0000 a	0.0000 a	0.5000 ab	1.0000 ab	0.4333 b
Stem + 0 Gy	1.2167 ef	0.8167 g	1.5000 cd	3.2500 bc	1.0500 cd
Stem + 10 Gy	0.7667 cd	0.2500 b	1.8333 d	6.4167 d	1.0333 cd
Stem + 30 Gy	1.1000 e	0.5333 def	1.0000 bc	2.3333 ab	1.1333 d
Stem + 50 Gy	0.0000 a	0.0000 a	0.5000 ab	1.5000 ab	1.1167 d
Cotyledon + 0 Gy	1.1000 e	0.5000 cdef	0.0000 a	0.0000 a	0.0000 a
Cotyledon + 10 Gy	1.1917 ef	0.6833 fg	0.0000 a	0.0000 a	0.0000 a
Cotyledon + 30 Gy	0.4000 b	0.2000 b	0.0000 a	0.0000 a	0.0000 a
Cotyledon + 50 Gy	0.6000 bc	0.3000 bc	0.0000 a	0.0000 a	0.0000 a
Media x Explants x Gamma Radiation					
MS + apical shoot + 0 Gy	1.6000 g	0.5000 bcd	2.0000 cd	6.0000 cdefg	1.4000 de
MS + apical shoot + 10 Gy	1.3167 efg	0.6500 cd	3.6667 e	10.000 g	2.2000 f
MS + apical shoot + 30 Gy	0.9333 cd	0.4000 bc	1.3333 bc	4.6667 bcdef	0.8333 b
MS + apical shoot + 50 Gy	0.0000 a	0.0000 a	0.0000 a	0.0000 a	0.0000 a
MS + stem + 0 Gy	1.4000 fg	1.2000 e	2.0000 cd	3.5000 abcde	0.9000 bc
MS + stem + 10 Gy	0.6000 bc	0.2667 ab	2.0000 cd	8.5000 fg	1.1667 bcde
MS + stem + 30 Gy	1.0000 cde	0.6667 cd	1.0000 b	2.6667 abcd	1.0667 bcd
MS + stem + 50 Gy	0.0000 a	0.0000 a	0.0000 a	1.0000 ab	1.3000 cde
MS + cotyledon + 0 Gy	1.2000 defg	0.6000 cd	0.0000 a	0.0000 a	0.0000 a
MS + cotyledon + 10 Gy	1.3500 efg	0.6000 cd	0.0000 a	0.0000 a	0.0000 a
MS + cotyledon + 30 Gy	0.8000 cd	0.4000 bc	0.0000 a	0.0000 a	0.0000 a
MS + cotyledon + 50 Gy	1.2000 defg	0.6000 cd	0.0000 a	0.0000 a	0.0000 a
WPM + apical shoot + 0 Gy	1.2333 efg	0.6333 cd	2.0000 cd	7.3333 efg	1.5000 e
WPM + apical shoot + 10 Gy	0.6000 bc	0.2667 ab	2.6667 d	6.6667 defg	1.1667 bcde
WPM + apical shoot + 30 Gy	0.2667 ab	0.2667 ab	1.6667 bc	6.6667 defg	0.8333 b
WPM + apical shoot + 50 Gy	0.0000 a	0.0000 a	1.0000 b	2.0000 abc	0.8667 b
WPM + stem + 0 Gy	1.0333 def	0.4333 bc	1.0000 b	3.0000 abcde	1.2000 bcde
WPM + stem + 10 Gy	0.9333 cd	0.2333 ab	1.6667 bc	4.3333 abcdef	0.9000 bc
WPM + stem + 30 Gy	1.2000 defg	0.4000 bc	1.0000 b	2.0000 abc	1.2000 bcde
WPM + stem + 50 Gy	0.0000 a	0.0000 a	1.0000 b	2.0000 abc	0.9333 bc
WPM + cotyledon + 0 Gy	1.0000 cde	0.4000 bc	0.0000 a	0.0000 a	0.0000 a
WPM + cotyledon + 10 Gy	1.0333 def	0.7667 d	0.0000 a	0.0000 a	0.0000 a
WPM + cotyledon + 30 Gy	0.0000 a	0.0000 a	0.0000 a	0.0000 a	0.0000 a
WPM + cotyledon + 50 Gy	0.0000 a	0.0000 a	0.0000 a	0.0000 a	0.0000 a

Remarks: numbers followed by same letters in the same column indicate no significant difference.

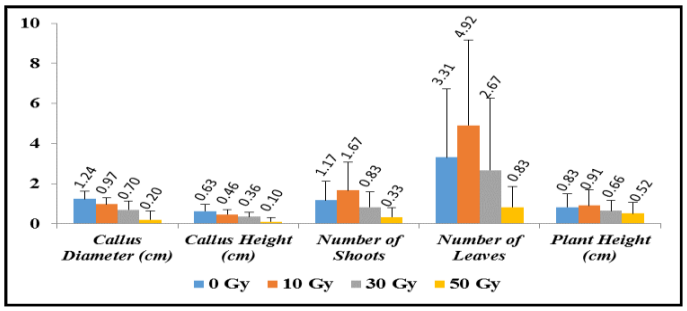

Fig 2. The effect of doses of gamma radiation on *in vitro* regeneration of loquat, 15 weeks after subcultures.

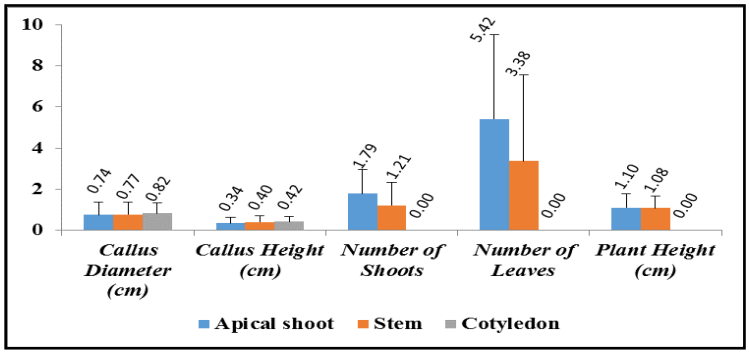

Fig 3. The effect of explants on *in vitro* regeneration of loquat, 15 weeks after subcultures.

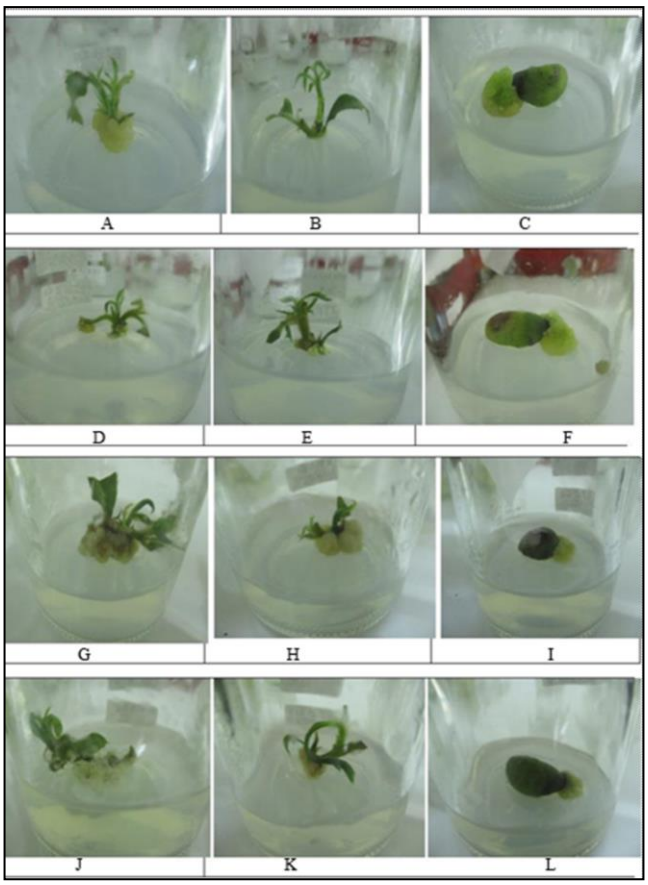

Fig 4. *In vitro* regeneration of loquat, 15 weeks after planting. (A) MS+10 Gy+Apical shoot; (B) MS+10 Gy+Stem; (C) MS+10 Gy+Cotyledon; (D) WPM+10 Gy+Apical shoot; (E) WPM+10 Gy+Stem; (F) WPM+10 Gy+Cotyledon; (G) MS+0 Gy+Apical shoot; (H) MS+0 Gy+Stem; (I) MS+0 Gy+Cotyledon; (J) WPM+0 Gy+Apical shoot; (K) WPM+0 Gy+Stem; (L) WPM+0 Gy+Cotyledon.

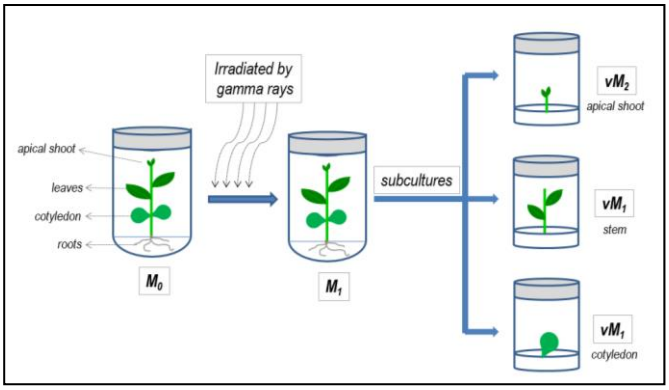

Fig 5. An overview of induced mutation and *in vitro* regeneration of *Eribotrya japonica.*

In this case, the MS medium produced more leaf numbers than WPM (Fig. 1). In this study, direct organogenesis was demonstrated by plantlets derived from shoot and stem explants. In addition to shoots, stem can be regarded as a potential source of explant. Although its ability to form a plantlet is not as high as shoots, it still produces a high number of buds, number of leaf and stem height for both planting media. Organogenesis is not directly indicated by the formation of callus in cotyledons (Fig. 3). Callus is a meristematic tissue as dedifferentiation formed. The callus formation is an important step in tissue culture. After that, we can add treatments for stimulation of new callus for roots and shoots formation. In this study, callus was produced in almost all cotyledons. Callus is formed from a complex interaction between explant, medium composition and environmental conditions during the incubation period. The callus has lignin that forms a strong and compact texture. In this study, the callus was yellowish and greenish colors (Fig. 4). A single application of auxin is generally sufficient to induce callus formation. However, recalcitrant seeds need more than one type of endogenous auxin simultaneously. Callus initiation is formed from differentiation of epidermal cells and sub epidermal areas. The multiplication of callus can be both embryogenic and non-embryogenic (Guedes et al., 2011). Callus induction is needed to generate somatic cell diversity and to regenerate these cells into somatic embryos.

In this study, there are some obstacles, e.g. explants browning and fungi contamination. However, the opportunities and challenges in mutation induction and loquat regeneration are still very open. The growth of shoots and loquat plantlet was quite fast. Callus is easily formed on several explants, so it can be more developed into plantlet through subculture on different media. In addition, some combination of media, hormones and radiation doses can be examined to create the best plantlet.

Materials and methods

Plant materials (explants)

The experiment was conducted in the Laboratory of Cibodas Botanical Garden. Loquat seeds obtained from Cibodas Botanical Garden collection and germinated through *in vitro* culture. After four months, the *in vitro* germination seedling were used as explant for *in vitro* propagation. The explants of *in vitro* propagation were divided into three group i.e. apical shoot (± 1 cm), stem between apical shoot and cotyledon (± 1,5-2 cm), cotyledon (Fig 5). The apical shoot consisted of the apical bud and leaves. The stem means a portion of plant stem with two nodes. Furthermore, the cotyledon is the rudimentary or modified leaf of the plant embryo within seed which is appears from a germinating seed.

In vitro seed germination

Loquat seeds were washed under running tap water with a detergent solution then soaked in Tween 80 for 15 min. In order to minimize fungal and bacteria contamination, seeds were treated with a solution of fungicide (Benlox) and bactericide (Agrept) for 20 min by gentle shaking. They were then disinfected in 70% ethanol for 1 min and then soaked for 20 min in 20% (v/v) NaOCl solution and rinsed three times with sterile distilled water. Seeds were cultured in a bottle containing 20 mL of MS medium (Murashige and Skoog, 1962) supplemented with 30 g/L sucrose and solidified with 6 g/L agar. The pH was adjusted to 5.8 before autoclaving at 121ºC and 1 atm for 20 min. The cultures were incubated under controlled conditions at 20ºC ± 2ºC, 16: 8 hours (light: dark). The germinated seeds were used for irradiation by gamma rays.

Mutation methods

After four months, *in vitro* germinated seedlings of loquat were irradiated by gamma rays using a Co-60 source in the Center for Application of Technology of Isotope and Radiation-Jakarta. The concentrations of gamma rays were 0, 10, 30 and 50 Gray.

In vitro culture and regeneration

Irradiated seedlings were excised into three parts: apical shoot, stem, and cotyledon. The subculture protocol for manipulation of *in vitro* culture was following Predieri (2001), which is apical shoot as vM_2 and stem and cotyledon as vM_1 (Fig. 5). Each part of explants was transferred to basal Murashige and Skoog (MS) medium enriched with BA 2 mg/L+NAA 0.1 mg/L and Woody Plant Medium (WPM) enriched with BA 2 mg/L+NAA 0.1 mg/L as regeneration

medium. The experiment was designed using randomized complete design with three treatments and three replicates. The cultures were incubated under controlled conditions at 20ºC ± 2ºC, 16: 8 (light: dark). Data were observed and recorded 15 weeks after subculture for callus diameter (cm), callus height (cm), number of shoots, number of leaves and plant height (cm). Data were statistically analyzed using the analysis of variance (ANOVA) and significance between means was tested by Duncan's multiple range tests.

Conclusion

The current study documented the protocol of induced mutation and *in vitro* regeneration for production of mutant loquat from the apical shoot, stems, and cotyledons. The effective dose of gamma rays for induced mutation of in vitro seedling of loquat was less than 30 Gy. MS media was better than WPM media, in order to regenerate mutant loquat. In general, the combination of MS media, apical shoot and 10 Gy showed better responses for five parameters, i.e. callus diameter, callus height, number of shoots, number of leaves and plant height.

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