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Irradiation effect on *in vitro* organogenesis, callus growth and plantlet development of *Gerbera jamesonii*

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ABSTRACT

The present work was carried out to study the effects of gamma irradiation on *in vitro* growth of explants, callus and the formation of shoots and plantlets. Irradiation is known to exhibit or inhibit the differentiation of cells and growth of plants *in vitro*, which helps in producing new plant varieties. Gamma irradiation is one of the physical mutagens that are widely used for mutation breeding. A gradual decline was observed in the number of shoots regenerated from irradiated petiole explants compared to control. Numbers of shoots regenerated from irradiated petiole explant cultured on Murashige & Skoog medium supplemented with 2.0 mg L^{-1} BAP and 0.5 mg L^{-1} NAA was reduced to 6.6±0.9 from 7.5±0.4 (control) when explants were exposed to 20 Gray of irradiation dose. Similar observation was reported on effects of gamma irradiation on *in vitro* propagated plantlets. Gradual decline was observed based on plant height as the dose of gamma irradiation increased. A significant decline was observed in the fresh weight of irradiated callus compared to control. In this case, growth responses of callus were strongly influenced by the radiation dose. The fresh weight of callus was reduced to 76.4±2.2% compared to 89.7±0.5% of control when callus tissues were exposed to 20 Gy.

Keywords: *Gerbera jamesonii*, ornamental, gamma irradiation, explant, callus regeneration, plant growth regulator, MS medium.

RESUMO

Efeito da irradiação na organogênese *in vitro*, crescimento de calos e desenvolvimento de plântulas de gerbera

O presente trabalho foi realizado para estudar os efeitos da radiação gama no crescimento *in vitro* de explantes de calos, e a formação de brotos e mudas. A irradiação é conhecida por induzir ou inibir a diferenciação de células e o crescimento das plantas *in vitro*, o que ajuda na produção de novas variedades vegetais. Radiação gama é um dos agentes mutagenicos que são amplamente utilizados para o melhoramento através da mutação. Um declínio gradual foi observado no número de brotos regenerados a partir de explantes de pecíolos irradiados comparado com o controle. O número de brotações regeneradas de explantes de pecíolos irradiados, cultivados em meio Murashige & Skoog, suplementado com 2,0 mg L^{-1} BAP e 0,5 mg L^{-1} ANA, foi reduzido de 7,5±0,4 (controle) para 6,6±0,9, quando explantes foram expostos a 20 Gray de irradiação. Observação semelhante foi relatada sobre os efeitos da radiação gama em plântulas propagadas *in vitro*. Um declínio gradual foi observado com base na altura da planta com o aumento da dose de radiação gama. Um declínio significativo foi observado no peso fresco de calos irradiados em relação ao controle. Neste caso, as respostas de crescimento de calos foram fortemente influenciados pela dose de radiação. O peso fresco de calos foi reduzido para 76,4±2,2% em comparação com 89,7±0,5% (controle) quando calos foram expostas a 20 Gy.

Palavras-chave: *Gerbera jamesonii*, planta ornamental, irradiação gama, explante, regeneração de calos, regulador de crescimento, meio MS.

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The use of *in vitro* culture methods for the selection of variant types in ornamentals has been documented for many years especially for flower color, plant morphology and also physiological characters. Induced variability does not seem to be different from that known to occur spontaneously. However, mutagen treatment could increase mutant frequency severely. Although some variants such as changes in flower color may emerge from spontaneous mutations at relatively high rates, mutation frequency of many useful traits is very low (Ibrahim, 1969). Mutagen treatments therefore, are outstanding importance for practical breeding purposes. According to Broertjes & Van Harten (1978), many ornamental species are suitable for mutation breeding, since flower color and other mutations can be produced without altering any other characters of the original ideotype.

In vivo and *in vitro* plantlet regeneration method could lead to the occurrence of variation through the new phenotype produced. Variation refers to the differences of genetic variation of cells whereby the characteristics of mother plant is delivered to the new plant. Most cultured plant cells could produce somaclonal variation which is another way of producing new and interesting plant phenotype through *in vitro* regeneration. Besides variation, propagation of new plantlets through *in vivo* and *in vitro* systems could also cause mutation and the effects of mutations could be observed through the new plant phenotype produced. Radiation is one of the physical factors that initiate mutations of plant cells when exposed to certain dosages. Morphological changes were observed when intact and *in vitro* plants were

exposed to radiation.

Radiation could initiate or inhibit the growth and differentiation of *in vitro* tissue cultured cells. Broertjes & Van Harten (1978) stated that through *in vitro* propagation, the effects of radiation were observed in the flower structures and colors of *Saintpaulia*, *Streptocarpus*, *Kalanchoe* and *Achimenes*. Through tissue culture system, not only plant organs, callus tissues could also be mutated when exposed to gamma radiation.

Gamma rays, X-rays, visible light and UV are all electromagnetic (EM) radiation. Electromagnetic radiations, have the energy level from around 10 kilo electron to several hundred kilo electron volts, and therefore are more penetrating than other radiation such as alpha and beta rays (Kovacs & Keresztes, 2002). Gamma rays belong to ionizing radiation and interact with atoms or molecules to produce free radicals in cells. These radicals can damage or modify important components of plant cells and have been reported to affect differentially on morphology, anatomy, biochemistry and physiology of plants depending on the irradiation level. These effects include changes in the plant cellular structure and metabolism such as dilation of thylakoid membranes, alteration in photosynthesis, modulation of the antioxidative system and the accumulation of phenolic compounds (Kim *et al*., 2004; Kovacs & Keresztes, 2002; Wi *et al*., 2005). Ionizing radiations are also used to sterilize agricultural products in order to increase their conservation time or to reduce pathogen when being traded from a country to another (Melki & Salami, 2008).

Mutation breeding influences morphological, physiological and plant cell behavior. Mutagenesis is a radiation process towards *in vitro* regeneration which is very useful for mutation induction in many plant species with high commercial values. In most ornamental plant species, mutation could be easily identified based on flower size, color and structure. Therefore, combinations of organogenesis and mutagenesis have the capability to increase variations in plants. Gamma radiation can be useful for the alteration of physiological characters (Kiong *et al*., 2008). The biological effect of gamma-rays is based on the interaction with atoms or molecules in the cell, particularly water, to produce free radicals (Kovacs & Keresztes, 2002). These radicals can damage or modify important components of plant cells and have been reported to affect differentially the morphology, anatomy, biochemistry and physiology of plants depending on the radiation dose (Ashraf *et al*., 2003). These effects include changes in the plant cellular structure and metabolism e.g., dilation of thylakoid membranes, alteration in photosynthesis, modulation of the anti-oxidative system and accumulation of phenolic compounds (Kovacs & Keresztes, 2002; Kim *et al*., 2004; Wi *et al*., 2006; Ashraf, 2009).

From the ultrastructural observations of the irradiated plant cells, the prominent structural changes of chloroplasts after radiation with 50 Gy revealed that chloroplasts were more sensitive to a high dose of gamma rays than other cell organelles. Similar results have been reported to be induced by other environmental stress factors such as UV, heavy metals, acidic rain and high light (Molas, 2002; Barbara *et al*., 2003; Quaggiotti *et al*., 2004). However the low dose irradiation did not cause changes in the ultra structure of chloroplasts. Irradiation of plants with high doses of gamma rays disturb the synthesis of protein, hormone balance, leaf gas-exchange, water exchange and enzyme activity (Esfandiari *et al*., 2008).

So far, there are few works reporting on the effects of irradiation in tissue culture system by previous researchers. Most of the plant species irradiated have very high importance and commercial values such as *Saintpaulia*, *Kalanchoe*, *Streptocarpus*, *Begonia*, *Dianthus caryophyllus*, *Chrysanthemum*, *Helianthus annus, Nicotiana tabacum*, *Phaseolus vulgaris* and *Daucus carota* among others. Irfaq & Nawab (2001) opened a new era for crop improvement and now mutation induction has become an established tool in plant breeding that can supplement the existing germplasm and improve cultivars in certain specific traits.

Not many reports were documented on the irradiation effects on *Gerbera* sp. and the study of irradiation effects on *G. jamesonii* is very limited. The present study is conducted to investigate the effect of gamma irradiation on shoot formation, plantlets and callus tissues of *G. jamesonii*.

MATERIAL AND METHODS

Seeds of *Gerbera jamesonii* were surface sterilized using 40% commercial bleach (clorox) for 20 min, followed by soaking in 70% alcohol for 1 min and finally rinsed of with sterile distilled water 3 times repeatedly. Subsequently, the seeds were germinated aseptically on MS (Murashige & Skoog, 1962) basal medium fortified with 30 g L^{-1} sucrose and 8.0 g L^{-1} technical agar. pH of the medium was adjusted to 5.8 prior to autoclaving at 121°C for 20 min. Cultures were maintained at 25±1°C with the photoperiod of 16 hours light and 8 hours dark. Seeds were germinated *in vitro* after 6-7 days in culture. Complete plants were obtained after 6-8 weeks. Leaves and petioles from these seedlings were utilized as source of explants.

Gamma radiation was provided from ^{60}Cobalt, 0026 Pool Irradiator with isotope model located at Physics Department, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia. Gamma radiation used for this experiment was fixed at doses of 10, 20, 30, 40, 50 and 60 Gray (Gy). The gamma dose rate was 0.204 Gy $second^{-1}$ at the time the experiment was conducted. Therefore, each exposure of the gamma radiation was fixed at 49, 98, 147, 196, 245 and 294 seconds.

In order to study the effect of gamma radiation on regeneration of *in vitro* shoots, petiole was used as source of explant. Explants were irradiated at 0-60 Gy and subsequently transferred and maintained in a fresh new MS medium supplemented with 2.0 mg L^{-1} BAP and 0.5 mg L^{-1} NAA. This medium was used since it is identified as the optimum medium for shoot regeneration *in vitro*. Response of irradiated explants was monitored and recorded after 8 weeks. As for the effect of gamma

irradiation on *in vitro* plantlets, eight-week-old *in vitro* plantlets regenerated from petiole explants cultured on MS medium supplemented with 2.0 mg L^{-1} BAP and 0.5 mg L^{-1} NAA were used as explant. Plantlets were exposed to Gamma irradiations at 0-60 Gy and later transferred to a fresh culture medium supplemented with the same concentration of growth regulator. After 12 weeks, irradiated plantlets were consequently acclimatized and transferred to the greenhouse. Growth and development of these plantlets were observed and recorded. Whereas, for the effect of gamma irradiation on callus tissues, fresh callus (2.0 g) was induced from leaf explants cultured on MS medium supplemented with 1.0 mg L^{-1} BAP and 2.0 mg L^{-1} 2, 4-D (optimum medium for callus induction). Induced callus was irradiated at 0-60 Gy and transferred to a fresh new medium. As for observation for irradiated callus tissues, at the end of 4-week culture period, total fresh weight of the callus was determined and morphological changes were observed and recorded. Fresh weight percentage of irradiated callus was calculated according to the following formula:

$$\frac{T1 - T2}{T3} \times 100$$ where:

T1 - Total fresh weight of irradiated callus (8th week) (mg)

T2 - Total initial weight of callus (mg)

T3 - Total initial fresh weight of callus (mg)

Subsequent to post irradiation, all cultures were incubated in the dark overnight and later placed in the culture room at 25±1ºC at 16 hours light and 8 hours dark.

Thirty explants were used in each treatment and experiments were repeated thrice. Experimental design was completely random and factorial with shoot formation, growth of plantlets and callus induction. All data were subjected to analysis of variance and comparison of mean was carried out using Duncan's Multiple Range Test (DMRT) and significance differences were determined at 5% level.

RESULTS AND DISCUSSION

A gradual decline was observed in the number of shoots regenerated from irradiated petiole explants compared to the control in all treatments. Number of shoots regenerated from irradiated petiole explants cultured on MS medium supplemented with 2.0 mg L^{-1} BAP and 0.5 mg L^{-1} NAA was reduced to 6.6±0.9 when explants were exposed to 20 Gray of irradiation dose (Table 1). Number of shoots regenerated was drastically reduced to 2.5±4.6 (Table 1, Figure 1c) when explants were exposed to 60 Gray of irradiation dose. However, abnormalities in the shoots formed from irradiated explants were observed when explants were exposed to Gamma irradiation at 30 Gy (Figure 1a), 40 Gy (Figure 1b), 50 Gy and 60 Gy (Figure 1c). From the results, it was observed that irradiated explants showed morphological and irradiation response in the formation of stunted and abnormal shoots. Kiong *et al*. (2008) reported that survival of plants to maturity depends on the nature and extent of chromosomal damage. Increasing frequency of chromosomal damage with increasing radiation dose may be responsible for less germinability and reduction in plant growth and survival.

For an effective use of mutation induction in plant breeding programms, the basic requirement is the analysis of radiosensitivity of the explant material. Leaves of *Rosa hybrida* subjected to increasing doses of radiation showed a decrease in regeneration capacity, which was completely suppressed at 100 Gy (Ibrahim, 1969). The lethal dose for 50% of the regenerating explants in irradiated explants was estimated to be 25 Gy. In mutation breeding of *G. jamesonii*, the dose chosen should result in the highest survival of irradiated explants and a low inhibition of the rate of production of new shoots would give the highest efficiency in recovering useful mutants (Laneri *et al*., 1990). Gamma rays imposed a significant impact on the shoot length of *Triticum aestivum* L. (Chauduri, 2002). Shoot length was declined up to 46% as the radiation dose increased (Borzouei *et al*., 2010). Norfadzrin *et al*. (2007) reported increasing gamma dosage decrease the germination and survival percentage of tomato and okra.

Jerzy & Zalewska (1996) irradiated

Table 1. The effects of gamma irradiation on *in vitro* regeneration of shoots, plantlets and callus of *Gerbera jamesonii* (efeitos da radiação gama sobre a regeneração *in vitro* de brotos, plântulas e calos de *Gerbera jamesonii*). Malaysia, Sultan Idris Education University, 2010.

Dose (Gy)	Irradiated petiole explant (number of shoots) (mean±SE)	Irradiated *in vitro* plantlets (number of shoots) (mean±SE)	Irradiated callus (total fresh weight) (%) (mean±SE)
0 (control)	$7.5 \pm 0.4_{a}$	$8.7 \pm 0.6_{a}$	$89.7 \pm 0.5_{a}$
10	$7.0 \pm 1.1_{b}$	$6.8 \pm 1.1_{b}$	$67.5 \pm 1.6_{b}$
20	$6.6 \pm 0.9_{b}$	$5.7 \pm 0.9_{b}$	$56.4 \pm 2.2_{c}$
30	$5.3 \pm 1.5_{b,c}$*	$5.4 \pm 1.2_{b,c}$	$44.3 \pm 0.8_{c,d}$
40	$5.0 \pm 0.8_{c}$*	$4.9 \pm 0.5_{c}$*	$29.4 \pm 0.6_{e}$
50	$3.8 \pm 2.3_{d}$*	$4.6 \pm 1.5_{c}$*	$23.1 \pm 1.7_{e}$
60	$2.5 \pm 4.6_{d}$*	$4.1 \pm 0.7_{c}$*	$10.2 \pm 0.5_{e,f}$

Means followed by the same letter in the same column did not differ according to Duncan's Multiple Range (DMRT) test at 5% significance level (médias seguidas pela mesma letra na mesma coluna não diferem de acordo com o teste de Duncan (DMRT) a 5%). *Formation of stunted and abnormal shoots (formação de brotos atrofiados e anormais).

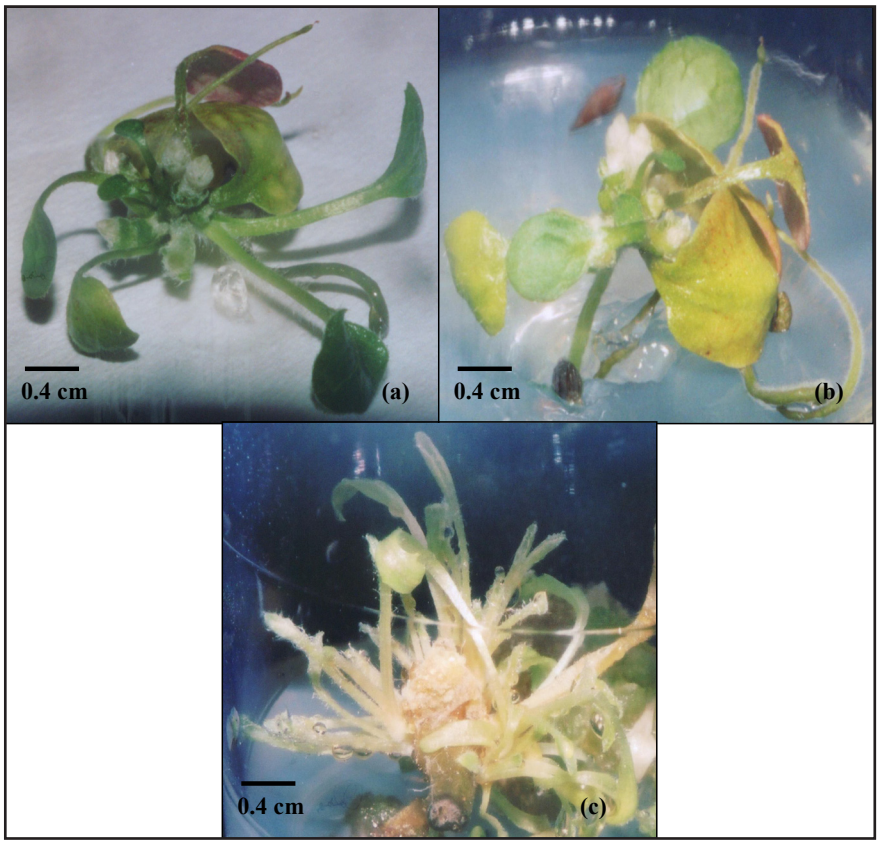

Figure 1. a) Regeneration of shoots from irradiated petiole explants of *Gerbera jamesonii* at 30 Gy; b) at 40 Gy and (c) at 60 Gy (a) regeneração de brotos a partir de explantes irradiados de pecíolos de *Gerbera jamesonii* a 30 Gy; b) a 40 Gy e (c) a 60 Gy). Malaysia, Sultan Idris Education University, 2010.

leaf explants from violet pink 'Richmond' of *Dendranthema grandiflora* with X-rays and Gamma rays at doses of 5 and 15 Gy. No undesirable mericlinal or sectorial chimeras were observed in the planting materials. This indicates that adventitious shoots regenerated from leaf explants usually arise from a single cell. In this way, the development of new plants consists of genetically homogenous tissues. Twelve new cultivars of *Rosa hybrida* were obtained after applying 15 Gy of X-ray irradiation at dose rate of 1.92 Gy/min (Ibrahim, 1969). Morphological abnormalities are of interest in ornamental horticulture, although some reverted to normal plants when plants were subcultured, indicating that the variations might be related to some temporary physiological disturbances (Raju *et al*., 1986).

Similar observation was reported on effects of gamma irradiation on *in vitro* plantlets of *G. jamesonii*. Gradual decline was observed based on plant height as the dose of gamma irradiation increased. Irradiated *in vitro* plantlets showed effect as the morphological aspects of the plantlets exhibited irradiation response. As the gamma dose increased, plantlets showed irregular and abnormal characters. Compared to control treatment with 8.7±0.6 cm, plantlet height was reduced to half with 4.1±0.7 cm when exposed to 60 Gy of irradiation dose (Table 1). Three-month-old irradiated plantlets were acclimatized and transferred to the greenhouse (Figure 2a-d). Kiong *et al.* (2008) found that radiation increases plant sensitivity to gamma rays and this may be caused by the reduced amount of endogenous growth regulators, especially the cytokinin, as a result of breakdown or lack of synthesis due to radiation.

Complete regeneration of *Dianthus caryophyllum* was achieved when petal explants were irradiated with Gamma radiation at 20 Gy and 40 Gy (Simard *et al*., 1992). Explants from various plant species have different sensitivity levels towards different irradiation doses. Apart from that, the activities of exogenous hormones in the culture media were also affected by gamma irradiation. Irradiation dose may have an effect on the effectiveness of the hormones and thus, this influenced the formation of shoots. Gamma irradiation may prohibit the auxin activities that may change the morphogenetic responses. Despite regeneration, the effect of irradiation on callus was also studied. A significant decline in the fresh weight of irradiated callus compared to the control was observed. In this case, growth responses of callus were strongly influenced by the radiation dose. The fresh weight of callus was reduced to 76.4±2.2% compared to 89.7±0.5% of control (Table 1) when callus tissues were exposed to 20 Gy gamma irradiation (Figure 3a). Total fresh weight of callus was further reduced to 64.3±0.8% (Figure 3b) and 59.4±0.6% (Figure 3c) when callus tissues were treated with 30 Gy and 40 Gy respectively. With increasing dose of gamma irradiation, the color of callus continued to darken and the tissues became brown in color and the structure of the tissue was relatively poor in contrast to the control. At 60 Gy, the fresh weight of callus tissues were reduced by half from the control with only 40.2±0.5% (Table 1). As the gamma irradiation dose increased, proliferation of callus tissues declined and total fresh weight was reduced compared to control.

Though the irradiation doses exposed to the callus tissues varies. Bajaj *et al*. (1970) reported similar results on the growth and development of *Phaseolus vulgaris* as the results obtained on irradiated callus of *Gerbera jamesonii*. According to Bajaj *et al*., (1970), the development of callus tissues declined when callus were exposed to 10-50 Gy) and drastically reduced when exposed to higher irradiation doses (200-300 Gy) while callus growth was lethal at 400 Gy of irradiation dose. Similar results were also obtained on irradiated callus of *Zea mays* (Moustafa *et al*., 1989), *Nicotiana tabacum*, *Antirrhinum majus* (Rao *et al*., 1976) and *Helianthus annus* (Omar *et al*., 1993). High irradiation dose (200-300 Gy) did not stimulate

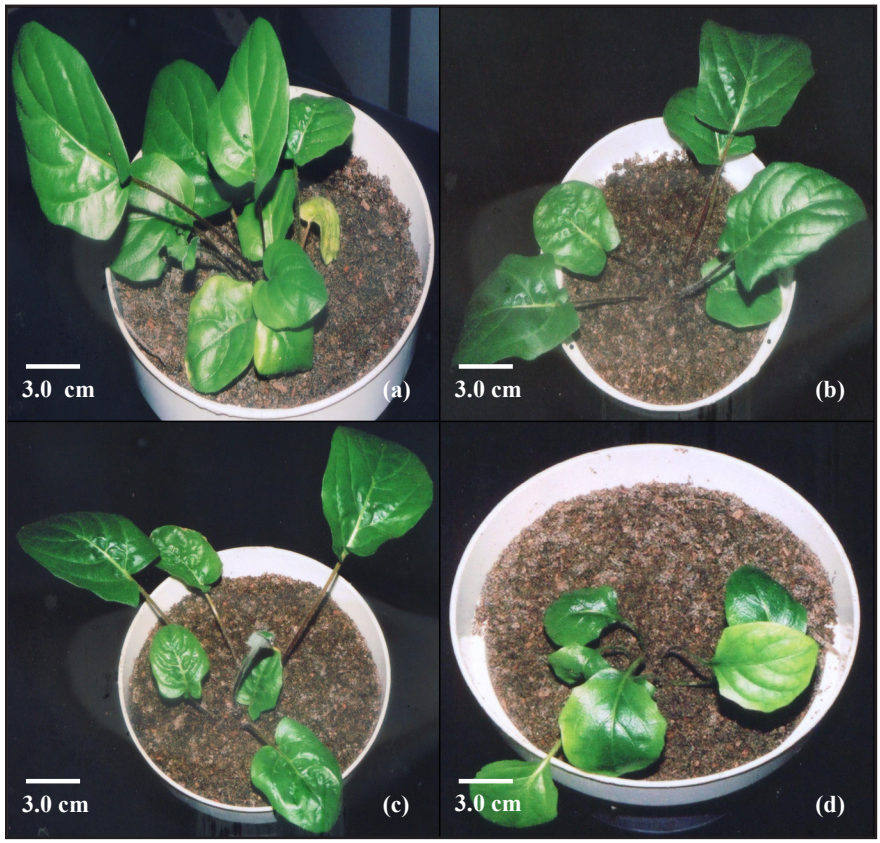

Figure 2. Three-month-old acclimatized plantlets; a) non-irradiated plantlets; b) irradiated at 10 Gy; c) irradiated at 20 Gy and d) irradiated at 30 Gy (plântulas aclimatadas aos três meses de idade; a) plântulas não irradiadas; b) irradiadas com 10 Gy; c) irradiadas com 20 Gy e d) irradiadas com 30 Gy). Malaysia, Sultan Idris Education University, 2010.

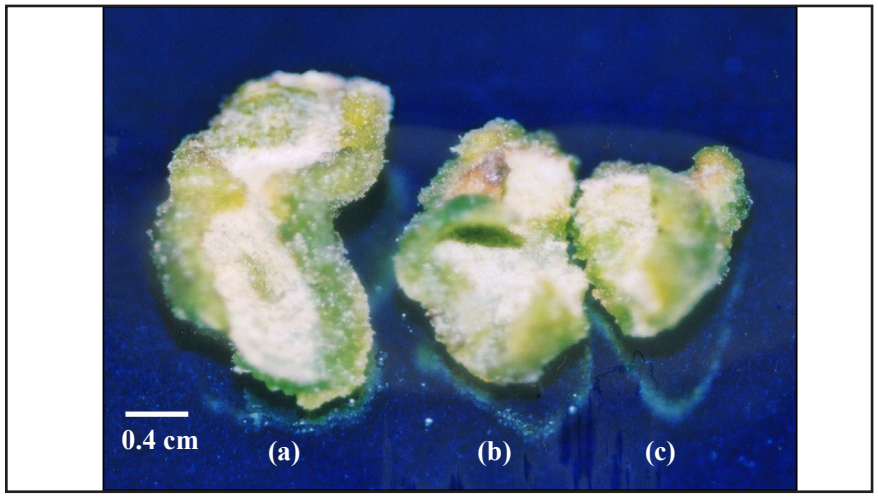

Figure 3. Irradiated callus obtained at a) 20 Gy; b) at 30 Gy and c) at 40 Gy (calos irradiados, obtidos em a) 20 Gy; b) 30 Gy e c) 40 Gy). Malaysia, Sultan Idris Education University, 2010.

callus growth of *Antirrhinum majus* and the callus failed to differentiate or form leaf primordial (Rao *et al*., 1976). The capability of callus tissues to differentiate and form shoots was diminished when the callus tissue was exposed to higher irradiation dose.

Physiologically, gamma irradiation has effect on the cell wall and the cell membrane. The decline of callus tissue growth is affected by the high irradiation dose exposed to the tissues. Thus, this will cause changes in the cell size or the callus tissues. Effect of irradiation dose not only prohibit water intake to the cells, it may also influence the synthesis of endogenous hormones. The reduction of callus growth may be caused by the lesser amount of endogenous hormones (auxin) in the explant.

Physiological symptoms in a large range of plants exposed to gamma rays have been described by many researchers (Kim *et al*., 2004; Kovacs & Keresztes, 2002; Wi *et al*., 2005). The symptoms frequently observed in the low or high dose irradiated plants are enhancement or inhibition of germination, seedling growth and other biological responses (Kim *et al*., 2000; Wi *et al*., 2005). The growth of *Arabidopsis* seedlings exposed to low-dose gamma rays (1-2 Gy) was slightly increased compared to control, while the seedling growth was noticeable decreased by the high dose irradiation of 50 Gy (Wi *et al*., 2006).

Many studies have been carried out on the sensitivity and inhibition of plant growth due to irradiation in various species. The damage caused by irradiation can be expressed at the metabolic level before they appear as growth retardation and death.

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