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In Vitro Mutation of Capsicum annuum L.var. Kulai by Gamma Radiation

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Abstract*: The present work was carried out to investigate the effects of gamma radiation on regeneration of Capsicum annuum L. var Kulai via in vitro. Seeds of C. annuum were irradiated with various doses of gamma ray (0, 20, 40, 60, 80, 100, 200, 300, 400, 500, and 600 Gy) emitted from the Caesium-137 source at the rate of 4.31 Gy per minute. Irradiated seeds grown on MS medium without hormone for hypocotyl and cotyledon preparation as explant for in vitro regeneration. Seed germination rate revealed significant variation between treatments, and seeds started to germinate between 6 to 17 days. Irradiated seeds between 0-60 Gy were observed to germinate in less than 10 days. All explants including hypocotyl and cotyledon were cultured on MS medium with different concentrations of BAP in combination with $AgNO_3$ to observe the response of these explants to different hormone concentrations. From the observation, calluses were induced in 90% of hypocotyl and cotyledon explants in all treatments. The characteristics of calluses were varied with greenish friable, greenish compact, yellowish watery, yellowish friable and yellowish compact. In other treatments, calluses were found in purple, bright yellow and yellowish orange. On the other hand, shoot regeneration was observed in treatment between 40-100 Gy. In conclusion, gamma radiation gave impact on seed germination, seedling growth performance, in vitro callus formation and shoot regeneration of Capsicum annuum var. Kulai.

Keywords* : Callus induction, Capsicum annuum, induced mutation, shoot regeneration.

I. INTRODUCTION

Capsicum annum L. is a short-live, evergreen perennial plant and a species of the genus Capsicum. It forms an important ingredient in most cuisine especially in Asian [1]. The high demands have brought about attempts in increasing *C. annuum* supply. In Malaysia, there are several varieties of *C. annuum* such as CB4, MC11, Kulai and Cilibangi [2]. However, *C. annuum* is known for its recalcitrance especially in tissue culture procedures. Recalcitrance refers to failure of plant cells, tissue or organs to respond well to in vitro culture. From the previous studies, there are three limiting factors that prevent the plant from responding which are morphogenic nature, formation of rosette shoots or ill-defined shoot buds and genotypic dependence [3]. Thus, this variety requires mutation to increase their production and characteristics, hence improving the overall plant performance. Mutation was introduced to induce new important traits concerning plant size, blooming period and fruit ripening, fruit colour, self-compatibility, self-thinning and resistance to pathogens [4]. Mutagenesis was also used for direct improvement of certain qualitative and quantitative characters of the plant. Induced mutation using gamma rays is one of the most effective techniques for morphological and genetically variability in plants mainly among those with limited genetic background [5]. Therefore, this study was carried out on *C. annuum* var. Kulai to determine the effects of gamma radiation on seed germination, seedling growth and in vitro regeneration.

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II. PROCEDURE

A. Seed Preparation

Seeds from healthy ripe fruits of *C. annuum* var Kulai were washed under running tap water followed by 5% (v/v) Tween 20 for 30 minutes and rinsed three times with distilled water. Seeds were then surface sterilized in 0.1% HgCl2 solution for 25–30 min, rinsed three times with sterile distilled water to remove any traces of sterilant. Seeds were placed on MS basal medium [6] containing 3% of sucrose and 0.2% agar.

B. Gamma Radiation Treatment

These seeds were exposed to gamma radiation at 0, 20, 40, 60, 80, 100, 200, 300, 400, 500, and 600 Gy from Biobeam GM 8000 irradiator machine located at Malaysian Nuclear Agency, Selangor. Untreated seeds (control) and treated seeds were sub-cultured into vials containing MS medium. Each treatment was replicated five times. The seeds were observed for day to germinate, and germination rate was calculated after two weeks of culture. Seedling height and root length were measured after two weeks of germination.

C. In vitro Plant Regeneration

Hypocotyls and cotyledons of *C. annuum* from sixteen-day-old seedlings were excised into pieces ranging between 2-3 mm in length and used as explant.

Each segment of hypocotyl was placed horizontally in each vial and was gently pressed onto the surface of the sterilized culture medium containing 3% of sucrose, 0.2% gelrite, 1-5 mg/L BAP in combination with 0-3 mg/L $AgNO_3$ and placed under fluorescent light with controlled temperature (22 ± 2°C) using 16 h photoperiod. Subculture of explants into new medium were done after three weeks of culture. Response of irradiated explants was monitored and recorded in two-week intervals.

D. Analysis of Result

The data were subjected to analysis of variance according to a randomized complete block design [7]. Data were analysed using Computer software Minitab version 16 and Statistical Package for the Social Science (SPSS). The assessment was done through qualitative and quantitative analysis by observing the seed germination rate (%), day for seed to germinate, height and root length of seedlings, weight (g) and characteristics of the callus formed.

III. RESULTS

A. Seed Preparation

From observation, similar results were observed on seed irradiated with 20-80 Gy with control treatment where 100% of seeds germinated after two weeks of culture (Table-I). Generally, *C. annuum* seeds started to emerge six or seven days after sowing [8] and this was proven in seeds from the control treatment. However, days to germinate of irradiated seed were significantly increased as the gamma doses increase. Seeds irradiated with 20 Gy, 40 Gy, and 60 Gy germinated in less than 10 days which were 7.6±0.548, 8.4±0.548 and 9.4±0.548, respectively. Seeds irradiated with 80, 100, 200, 300, 400 and 500 Gy germinated in more than 10 days (10.6, 11.6, 13.0, 13.8, 14.8, 15.8 and 16.8 days, respectively). The longest day to germinate was observed on seed irradiated with 600 Gy (16.8±0.447). Although the days to germinate varied in different treatments, almost 100% germination was achieved in treatment 100 Gy and below after two weeks of culture. However, germination frequencies decreased gradually from treatment 100 Gy (98%) to 600 Gy (19%).

Table-I: Effect of Gamma radiation on Seed germination rate and Day to Germinate

Gamma dose (Gy)	Day to Germinate	Germination rate (%)
0	6.20a	100a
20	7.60b	100a
40	8.40c	100a
60	9.40d	100a
80	10.60e	100a
100	11.60f	98a
200	13.0g	80b
300	13.8h	63c
400	14.8i	46d
500	15.8j	30e
600	16.8k	19f

The height of seedling, root length of control and irradiated seedling showed a decreasing pattern towards the increase of gamma ray doses after two weeks of culture (Table-II). The height of seedling germinated from 0 Gy was 4.0 cm and for irradiated seed, the highest plant was achieved in treatment 20 Gy (3.8 cm) which was slightly lower than non-irradiated seeds. The decreasing trend was observed from the rest of the treatments. It was found that after 14 days, the seedlings from 600 Gy reached only 0.25 cm in height.

Table-II: Mean Seedling Height (cm) and root length as effected by different Doses of Gamma Radiation

Radiation dose (Gy)	Seedling Height (cm)	Root Length (cm)
0	4.00a	2.80a
20	3.80b	2.50b
40	3.30c	2.00c
60	2.50d	1.60d
80	1.86e	1.40e
100	0.84f	1.20f
200	0.60g	1.16g
300	0.45h	1.40h
400	0.40i	1.20i
500	0.34i	1.20i
600	0.25k	1.20i

B. Callus Induction

In this study, further investigation was carried out on cotyledon and hypocotyl from six selected treatments which were 0 Gy, 20 Gy, 40 Gy, 60 Gy, 80 Gy and 100 Gy. Samples from 200 Gy until 600 Gy were discarded since no significant development was observed after irradiation. The source for explants was difficult to obtain because they responded negatively on its early development. Explants from selected treatments were cultured for callus and shoot induction on media supplemented with different concentrations and combinations of BAP and $AgNO_3$. In order to determine the performance of these explants, weights of callus were measured after 14 days of culture on selected media (Table-III). From the observation, callus initiated from explants of 20 Gy showed no significant different compare to control treatment (0 Gy). The weight of calluses from treatment 20 Gy were 0.33±0.03a and 0.41±0.04a in both types of explants, hypocotyl and cotyledon respectively. Weight of calluses were decreased significantly as gamma dose increase.

Table-III Weight of callus initiated from hypocotyl and cotyledon explants of seeds irradiated with 0, 20, 40, 60, 80, and 100 Gy after culture for 14 days.

Gamma dose (Gy)	Hypocotyl (gram)	Cotyledon (gram)
0	0.35±0.06a	0.43±0.04a
20	0.33±0.03a	0.41±0.04a
40	0.29±0.03b	0.37±0.03b
60	0.28±0.04b	0.32±0.03c
80	0.23±0.04c	0.30±0.04d
100	0.18±0.03d	0.26±0.04e

Means with different letter(s) in column are different between treatments (p=0.05)

The formation and characteristics of callus were observed and recorded in Table-IV (hypocotyl explant) and Table-V (cotyledon explant).

Percentage of callus formation in hypocotyl explants was in the range of 90–96% while in cotyledon explants 100% of callus was regenerated. Their characteristics observed were classified into several groups based on colour and form of callus namely greenish friable, greenish compact, greenish watery, yellowish friable, yellowish compact and yellowish watery. There were calluses that showed more than one characteristic in few treatments (Fig. 1 & Fig. 2).

Table-IV: Callus formation and characteristic induced from hypocotyl explant of *C. annuum* after two weeks of culture in MS medium supplemented with 20 different combinations of $AgNO_3$ and BAP concentrations.

Radiation dose (Gy)	Callus Regeneration		
	Treatment	Growth rate (%)	Characteristic
0	$T_{0\text{-}4H}$ $T_{0\text{-}7H}$ $T_{0\text{-}17H}$	92 92 92	Yellowish, friable Yellowish, watery Greenish, compact
20	$T_{20\text{-}12H,}$ $T_{20\text{-}20H}$	90	Greenish, compact
40	$T_{40\text{-}9H}$	90	Greenish, friable
60	$T_{60\text{-}16H,}$ $T_{60\text{-}19H}$	90 90	Yellowish, watery Yellowish, friable
80	$T_{80\text{-}17H}$	96	Greenish, friable
100	$T_{100\text{-}10H}$	92	Greenish, compact

Table-V: Percentage of callus formation and characteristics of callus induced from cotyledon explant of *C. annuum* after two weeks of culture in MS medium supplemented with 20 different combinations of $AgNO_3$ and BAP concentrations.

Dose (Gy)	Callus Regeneration		
	Treatment	Growth rate (%)	Characteristic
0	$T_{0\text{-}3C}$, $T_{0\text{-}7C}$, $T_{0\text{-}11C}$, $T_{0\text{-}15C}$ $T_{0\text{-}19C}$, $T_{0\text{-}20C}$ $T_{0\text{-}6C}$, $T_{0\text{-}10C}$ $T_{0\text{-}14C}$, $T_{0\text{-}18C}$ $T_{0\text{-}8C}$	100 100 100 100 100 100	Yellowish, friable Yellowish, friable Yellowish, friable Greenish, compact Greenish, compact Greenish, friable
20	$T_{20\text{-}6C}$, $T_{20\text{-}7C}$, $T_{20\text{-}10C}$, $T_{20\text{-}14C,}$ $T_{20\text{-}18C}$, $T_{20\text{-}19C,}$ $T_{20\text{-}9C}$, $T_{20\text{-}17C}$ $T_{20\text{-}15C}$, $T_{20\text{-}20C}$	100 100 100 100 100 100	Greenish, compact Greenish, compact Greenish, compact Greenish, friable Greenish, friable Yellowish, friable
40	$T_{40\text{-}1C}$, $T_{40\text{-}17C}$ $T_{40\text{-}2C}$, $T_{40\text{-}18C}$ $T_{40\text{-}20C}$ $T_{40\text{-}3C}$, $T_{40\text{-}11C}$ $T_{40\text{-}15C}$, $T_{40\text{-}19C}$ $T_{40\text{-}4C}$, $T_{40\text{-}12C}$ $T_{40\text{-}16C}$	100 100 100 100 100 100 100	Greenish, friable Greenish,compact Greenish, compact Yellowish, friable Yellowish,friable Yellowish, compact Yellowish, compact
60	$T_{60\text{-}5C}$, $T_{60\text{-}7C}$ $T_{60\text{-}17C}$ $T_{60\text{-}6C}$, $T_{60\text{-}10C}$ $T_{60\text{-}11C}$ $T_{60\text{-}12C}$	100 100 100 100 100	Greenish,friable Greenish, friable Greenish,compact Yellowish, friable Yellowish, compact
80	$T_{80\text{-}12C}$ $T_{80\text{-}14C}$ $T_{80\text{-}15C}$, $T_{80\text{-}19C}$ $T_{80\text{-}12C}$	100 100 100 100	Yellowish, compact Greenish, compact Yellowish, friable Yellowish, friable
100	$T_{100\text{-}7C}$, $T_{100\text{-}8C}$ $T_{100\text{-}12C}$ $T_{100\text{-}15C}$ $T_{100\text{-}14C}$	100 100 100 100	Greenish, friable Yellowish, compact Yellowish, compact Greenish, compact

*H: hypocotyl, C: Cotyledon

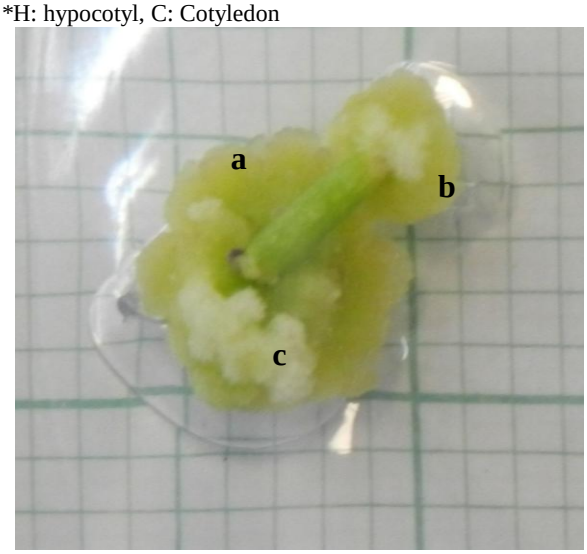

Fig. 1. Colour and morphology of callus induced from 40 Gy-hypocotyl ($T_{40\text{-}3H}$) culture on MS media supplemented with combination of 3.0 mg/L $AgNO_3$ and 1.0 mg/L BAP; Yellowish compact (a) Yellowish friable (b) and White friable (c).

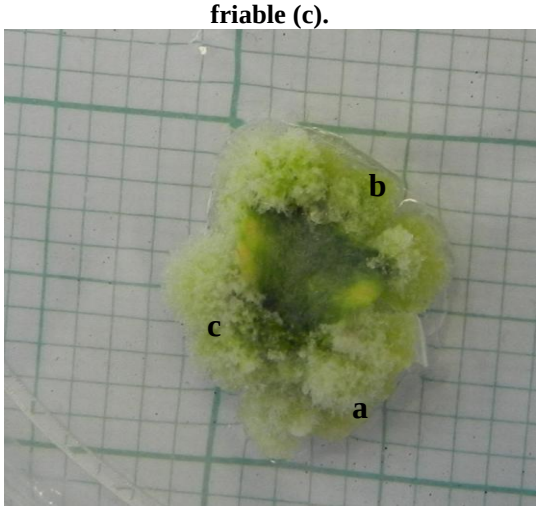

Fig. 2. Colour and morphology of callus induced from 60 Gy-cotyledon ($T_{60\text{-}7C}$) cultured on MS media supplemented with combination of 3.0 mg/L $AgNO_3$ and 2.0 mg/L BAP; White friable (a) Greenish compact (b) and Greenish friable (c).

C. Shoot Regeneration

Shoot regeneration of *C. annuum var*. Kulai was observed in several treatments meanwhile others, initiated callus and few shoots regenerated from callus in all 20 treatments of different concentrations and combinations of BAP and $AgNO_3$. Shoot regeneration was observed from cotyledon explant from treatment $T_{40\text{-}9H,}$ $T_{60\text{-}19C}$, $T_{80\text{-}19C}$ and $T_{100\text{-}19C}$ on MS media. Meanwhile for the hypocotyl culture, shoot regeneration was observed in treatment $T_{80\text{-}13H}$. Fig. 3(a) below shows the shoot regenerated from cotyledon of seeds irradiated with 80 Gy-cotyledon ($T_{80\text{-}13C}$) and Fig. 3(b) shows hypocotyl obtained from seeds irradiated with 40 Gy-hypocotyl ($T_{40\text{-}9H}$).

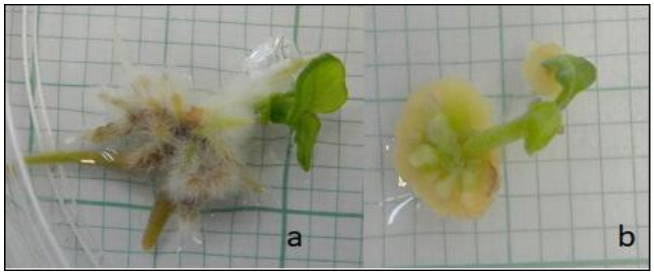

Fig. 3. Shoot regeneration in cotyledon explant obtained from $T_{80\text{-}13C}$ (a); Shoot regeneration in hypocotyl explant obtained from $T_{40\text{-}9H}$ cultured on MS medium (b).

IV. DISCUSSION

A. Seed Germination and Survival Rate

Seed germination and survival rate of irradiated seeds showed that the gamma ray affect both parameters. These results are in accordance with the findings of previous researchers who reported that the seed germination potential of different crops decreases when the radiation dose is decreased such as the case in wheat [9] and maize [10], [11]. Similar study on *C. annuum* by [12] reported that 20 and 60 doses have led to the lowest response in emergence index, germination rate and total germination. Meanwhile, [13] reported that the emergence of the control seedlings of *C. annuum* (44.44%) was similar to that of 300, 400 and 500 Gy with percentages of 45.56% to 38.89% at 20 days after planting; and germination in higher doses was less than 20%.

In general, there were no favorable stimulative effects from the gamma irradiation doses of 20-600 Gy on germination, seedling height and root length of *C. annuum* var. Kulai. Gamma irradiation has adversely affected the seed germination and development of seedlings when compared to control treatment.

High doses of gamma radiation would kill the plant because mutagen can give direct negative effects on plant tissue where many mutations can be lethal, yet low irradiation doses only affects several traits. The effects due to cytological changes include chromosomal damages, inhibited mitotic division, degeneration of nuclei and cell enlargement [14]. These effects eventually decreased seed germination and seedling growth.

B. Callus Induction and shoot regeneration

Shoot regeneration occurs when enhanced polyamines affect multiple shoots bud induction and this could be attributed to their stimulatory effect on cell division or inhibitory effect on production of ethylene [15]. Low concentration of BAP (3.0 mg/L BAP) treatments only produced one shoot while higher concentration of BAP (4.0 – 5.0 mg/L BAP) treatments produced more shoots. In the previous study on *C. annuum* L. by [16], it was found that higher concentration of BAP is effective for shoot multiplication in shoot tip explants. Similar observation was also found in other plants such as Jatropha [17], pineapple [18] and rockmelon [19]. While [20] determined that higher concentration of BAP (>2.0 mg/L) would not be effective in shoot regeneration. Suitable plant growth regulator of cytokinin especially BAP was difficult to determine. Similarly, it is also reported by [20] that it was difficult to initiate shoot as there was no direct shoot regeneration observed. The shoot is usually produced in the intermediary callus phase.

Several factors that may contribute to shoot initiation and further development of shoots such as the position of explants from the seedling, age of explants and types of explants. The study by [21] identified that explants from the basal position gave an advantage to develop shoots. While topophysic also affects the in vitro regeneration as reported by [22]. In order to avoid topophysic, explants of culture need to be chosen from the middle part of the sources.

In this study, three concentrations of $AgNO_3$ were used in the induction media. In some species, $AgNO_3$ improved callus proliferation [23] while for other species its enhanced shoot regeneration [24], [25]. However, in this study, $AgNO_3$ showed no significant effect on in vitro culture of *Capsicum annum* var. Kulai.

V. CONCLUSION

It can be concluded that gamma radiation has affected the seed germination and development of *C. annuum* seedlings. While for the in vitro study, various observations on shoot regeneration and callus induction were obtained. The present finding indicated that among these two types of explants, cotyledon induced more shoots than hypocotyl. Callus obtained can be differentiated by four types which were greenish friable callus, greenish compact callus, yellowish friable callus and yellowish watery callus. However, further investigation needs to be conducted to verify the variation caused by gamma radiation.

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