

Improved Regeneration, Acclimatization and Shoot Cutting Production of “Gebang” Cassava Derived from Irradiated *In Vitro* Shoots

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Abstract

Gebang is an Indonesian local genotype which has been selected as superior genotype for low amylose cassava. Prior to induction of new mutants of this genotype, series of research have been conducted to improve regeneration and acclimatization as well as shoot cuttings production of irradiated *in vitro* shoots. Four dosage treatments of gamma ray irradiation i.e. 0, 0.2, 1.0, 1.5, 2.0 krad were applied to 32 *in vitro* shoots multiplied from apical shoots of plants in the field. The highest multiplication rate and acclimatized shoots were obtained from shoots irradiated with 0.2 krad multiplied on various level of BAP added on MS medium and resulted that MS supplemented with 1 mg/L BAP was the best medium. The phenotypic variation was observed in shoots irradiated with 1.0, 1.5 and 2.0 krad, while that irradiated with 0.2 krad performed normal appearances. Subsequent production of propagated young stem cuttings so called “ratooning system” decreased after the second cycle of propagation especially in the survival rate of that irradiated with 0.2 krad. The findings lead to the opportunity to produce cassava propagules derived from irradiated *in vitro* culture at a higher amount using Gebang genotype as a model.

Keywords: cassava Gebang, irradiated shoot, young stemcutting/ratooning, acclimatization, *in vitro*

Introduction

Cassava (*Manihot esculenta* Crantz) is vegetatively propagated through stem cutting. It is one of the most important root crops in the world. Cassava is the third largest tropical crops used as a staple food especially in Africa, South America and Asia (Stupak *et al.*, 2006). As the roots yielded from the plants, cassava was feed over 500 million people worldwide. According to Tonukari (2004), the need of cassava as raw materials in developing countries increases each year by 2.0% for food and 1.6% for feed. It is predicted that the total production from all current production level will be projected to around 168 million tons by the year of 2020 (Aerni, 2006). In Indonesia, the crop is utilized as staple food and mostly grown in marginal land.

Important factors in traditional breeding of cassava have limitations associated with the production and utilization of the crop. High level of heterozygote in cassava could naturally happen from a cross pollination due to the differences in

maturity of male and female flowers. On the contrary, there are only a few numbers of cassava cultivars which success transformed to date. Therefore, it is necessary to apply another technique to improve their genetic variation that can complement with the conventional methods. However, *in vitro* culture technique can be the best solution to overcome those of conventional breeding problems.

In vitro plant breeding can be carried out through somaclonal variation from tissue culture which is common to create genetic variation. The variation can be improved by using either physical treatment such as the application of ionizing radiation or chemical mutagens (Collin & Dix, 1990). These techniques have been applied in several crops such as rice (Gao *et al.*, 1992), sugar beet (Alikamanoglu, 2002) and sweet potato (Shin *et al.*, 2011). By such treatments, new clones can be expectedly produced as new sources of variation to increase plant genetic quality (van Harten, 1998). To achieve this idea, the establishment of important factors related to *in vitro* plant breeding such as regeneration and acclimatization as well as shoot stem cutting production is very crucial requirement.

Gebang is one of the Indonesian local genotypes of cassava which has been selected as superior

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genotypes as low amylose cassava (Priadi & Sudarmonowati, 2006). The establishment of genetic variation through mutation treatments would open further development of these genotypes addressed as food and raw materials for cassava/starch to cope with high demand of industries in Indonesia.

Objectives of the research were to improve regeneration and to conduct acclimatization as well as shoot cutting production of irradiated shoot of Gebang genotype to induce new mutant.

Materials and Methods

Plant Materials. The *in vitro* shoot cultures of Indonesian cassava Gebang genotype were isolated from apical shoot plants collected from the cassava collection garden at the Research Center for Biotechnology, Indonesian Institute of Sciences (LIPI). *In vitro* shoot cultures were prepared by subculturing nodal segments in new MS (Murashige and Skoog) medium containing 1.0 mg/L BAP alternating with MS hormone-free medium. The irradiation treatment of *in vitro* shoot cultures were conducted in the National Nuclear Energy Agency (BATAN), Indonesia.

Sterilization Procedure. Surface sterilization of apical shoots was conducted under running tap water for 10–15 min. Subsequently soaking in 4% Dithane for 30 min, and in 0.2% Agrept (a bactericide) for 30 min. That sterilization was placed on a shaker with 80 rpm. The sterilized apical shoots were then rinsed with aquadest several times, then in 70% ethanol for 5 min and in 0.1% HgCl₂ for 3 min. Final rinsing was conducted with sterilized aquadest for 3–5 times.

Somaclonal Variation Using Gamma Ray Irradiation. The submerged *in vitro* shoot collected from apical shoots cultures were exposed with gamma ray irradiation treatments at dosages of 0, 0.2, 1.0, 1.5, and 2.0 krad. Afterward the irradiated shoots were transferred to MS medium free-hormone and incubated in room culture with 16 h photo periodicity at 25°C temperature.

Regeneration and Multiplication of Irradiated Shoots. All irradiated shoots were regenerated and multiplied by subculturing their nodal segments in MS medium containing 1.0 mg/L BAP. Subculturing was repeated every 1–2 months for three cycles. The shoot numbers derived from the three multiplication

cycles and visual performances were compared among them for evaluation. The survive cultures resulted from either sub lethal or higher level dosage were maintained and evaluated for acclimatization step.

Acclimatization of Regenerated Plantlets. Acclimatization of plantlets from regenerated shoots was conducted by growing the plantlets on autoclaved soil, compost and sand in ratio of (1:1:1). Plantlets were carefully removed from agar media followed with washing the roots with aquadest. Number and length of roots, number of leaves and the height of plantlets were measured. The roots were then dipped in concentrated solution of Rootone prior to planting. The plantlets were then sprayed with a fungicide (Dithane) at a concentration of 45 g/L and covered with transparent plastic bags. Survived plants in polybags kept in the green house were transferred to the field after approximately 3 months in the green house.

The Multiplication/Propagation of Irradiated Shoot Cutting in the Field. The source stem propagation was taken from three surviving mother plant clones of irradiated Gebang with 0.2 krad dosages using modified ratooning technique developed by N'Zue *et al.* (2006) and conducted every 3–6 month until three cycles. Ratooning was conducted by cutting cassava stem less than 10 cm or 3–5 axilar nodes before their time of harvesting (commonly every 6 months) and let them recovery which was signed by regrowth of that cassava stem. The stems were planted at Experimental Station of Research Center for Biotechnology, Indonesian Institute of Sciences (LIPI). The height of three motherplant clones before they were applied ratooning technique and the survival rate of motherplant clones resulted from three ratooning cycles were compared.

Results and Discussion

The Multiplication Rate of Irradiated Shoot Cultures

The number of multiplied shoot multiplication cultures of Gebang plantlets from both 0.2 krad and 1.0 krad dosages were higher than those of other dosages on recovery step (Table 1). The leaves and stem abnormalities, and dwarf which commonly happen after irradiation treatments was

shown until two subculture cycles especially on *in vitro* shoot exposed with gamma ray irradiation at 1.0, 1.5, and 2.0 krad (Figures 1a-c). Moreover, of that 1.5 and 2.0 krad, *in vitro* shoots died in the first and second subculture cycles, respectively (Table 1 and Figures 1b-c). The best response of those treatments was found in plantlets irradiated with 1.0, 1.5 and 2.0 krad having abnormal stems and leaves except plantlets irradiated with 0.2 krad showing normal plantlets. This finding is in consistent and in line with Puchooa (2005) working with irradiated *Anthurium*. A boosting effect of the 0.5 krad dosage was indicated on the phenotypic performance of *Anthurium* plantlet derived from the *in vitro* leaf tissues. However, for the direct use of mutants as new cultivars in practical breeding, mutants should not have any unwanted mutations or abnormalities (Shin *et al.*, 2011). Therefore, mutants have to be selected from recessive mutants *i.e.*; reduced viability, growth abnormalities and reduced fertility in order to get only the desired mutants (Puchooa, 2005).

Of all shoots derived from two subculture cycles, the shoots with 0.2 krad and 1.0 krad showed higher response of multiplication than other dosages. Interestingly, the phenotypic of shoots with 0.2 krad irradiated shoots seemed to be normal shoots compared to those irradiated at 1.0 krad. Meanwhile, 1.5 and 2.0 krad irradiated shoots tend to inhibit their shoot growth (Table 1 and Figure 1b-c). This finding was consistent and in accordance with Ahloowalia & Maluszynski (2001) who reported that callus induced from plant *in vitro* cultures were much more sensitive to radiation treatment. They would turn to necrotic and lose their regenerative capacity on the higher dosage exposure. Furthermore, the limited number of available reports suggests that callus cultures require much lower doses in range of ± 0.2 to 0.5 krad than stem cuttings or seeds; with relatively higher doses (150 to 200 krad). However, the response of *in vitro* plants to radiation treatment is varied and depended on the condition and the genotype of that *in vitro* plant.

Acclimatization of Plantlets

The irradiated plantlet conditions such as number of roots, length of roots, the number of leaves and height of plantlets regenerated in various MS medium supplemented with plant growth regulators NAA and BAP were analysed before they were

Table 1. The number of shoots multiplication cultures of Gebang plantlets after gamma ray irradiation treatments.

Gamma ray (krad)	Number of shoots multiplication after irradiation treatments (cycles)			
	0	1	2	3
0	8	9	30	30
0.2	5	25	146	157
1.0	3	3	24	83
1.5	8	10	13	0
2.0	8	3	0	0

Note: This multiplication data were taken by disregarding the source number of irradiated plantlet.

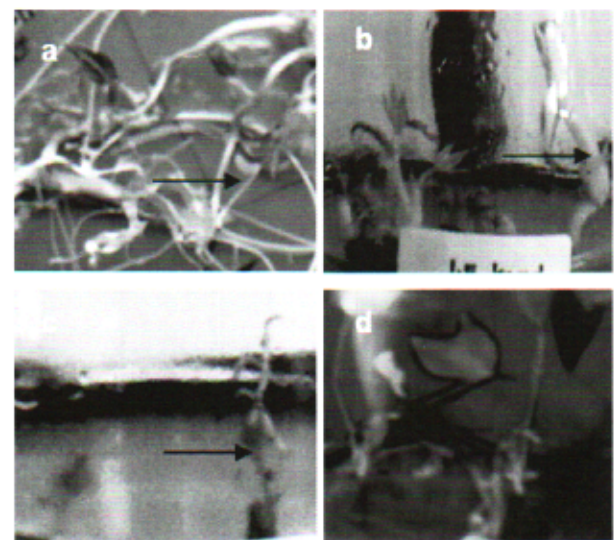


Figure 1. The phenotypic performance of irradiated cassava shoots after two subculture cycles: a. 1.0 krad derived shoot with long stem; b. 1.5 krad derived shoot with big stem and abnormal leaves; c. 2.0 krad derived shoot with very short leaves (tends to die); d. 0.2 krad derived shoot looking like a normal plantlet. (Note: arrows in figures showed the abnormality performances of plantlets).

acclimatized in the greenhouse. The results showed that there was a significant different ($P < 0.05$) of plantlet characters in terms of number of roots and plantlet height regenerated on various MS medium supplemented with NAA and BAP (Table 2 and Figures 2a-f). Furthermore, the highest of number roots was obtained from plantlet regenerated on MS medium supplemented with 1.0 mg/L of NAA and 0.5 mg/L of BAP meanwhile plantlet regenerated in MS medium supplemented with 1 mg/L of BAP showed the highest stem of all treatments. Despite plantlet regenerated on MS medium supplemented with 1.0 mg/L of NAA and 0.5 mg/L of BAP performed a well-plantlet condition particularly their

number of roots prior to acclimatization, however there were no survival plants obtained from those plantlets after months acclimatized in the greenhouse (Table 3). Meanwhile, plants regenerated on MS hormone-free medium followed with addition of 1.0 mg/l of BAP in MS medium were survived. However, their survival rate of individual plants was low. It indicated that many factors influenced plantlets acclimatization such as the genotype of plants and plantlets condition included the number of roots, leaves and the height of plantlets (Table 2) as well as growing media composition and humidity. This result supports Priadi & Sudarmonowati (2006) work which concluded that Gebang derived from somatic embryos seems to be weaker than Iding as less survived plants were obtained prior to acclimatization.

Data of survival individual plants regenerated on various MS medium supplemented with plant growth regulators before and after three months of plantlets acclimatization is presented in Table 4 and Figure 2. Most plants showed high level of survival rate after one month during acclimatization but they seemed to be very susceptible to many factors especially high humidity. Hence, the observation of survival plantlets was conducted after the third months of plantlets acclimatization.

The Production of Stem Propagation of Irradiated Cassava in the Field

The three irradiated cassava plants (mother plant clones) with 0.2 krad irradiated shoots were successfully transferred in the field. To increase the number of mutant resulted from irradiated cassava cultures, the mutant selection need to be done combined with vegetatively propagated stems (Donini *et al.*, 1998). Therefore, the vegetatively propagated stems were conducted by ratooning those three mother plant clones every 3–6 months for three cycles. Before ratooning, the height of the three clones was measured. After three ratooning cycles, it showed that the height of three clones tend to be stable except for the third clones which showed a decrease after three propagation cycles (Figure 3). This could happen due to the decrease of plant ability to grow normally as the effect of higher frequencies of propagation. The result indicated that stem characteristics tended to small, had dense nodul segment and many branches. This finding

was in accordance with Muthusamy *et al.* (2007) who reported that the plant characteristics such as the plant height and number of branches influenced on the plant growth capability after mutagenic treatments.

Table 2. Plantlet characters regenerated on various MS medium supplemented with hormones prior to acclimatization.

Medium	Number of Roots *	Length of roots (cm) Ns	Number of leaves Ns	Height of plants (cm) *
Ms 0	2.33 ± 1.15 b	4.00 ± 1.00	2.00 ± 1.00	5.00 ± 1.00 ab
1/2Ms	2.50 ± 0.70 b	4.00 ± 0.00	2.00 ± 1.41	3.00 ± 1.41 a
MS+1 mg/L NAA+0.5 mg/L BAP	6.33 ± 0.58 a	3.67 ± 1.15	1.67 ± 1.15	5.67 ± 1.15 ab
MS+1 mg/l BAP	3.00 ± 0.87 b	4.41 ± 0.50	3.00 ± 1.12	6.44 ± 1.67 b

Note: Means ± of standard deviation within column followed by different letters in each column are significantly different at P≤0.05 by ANOVA.



Figure 2. The acclimatization of irradiated cassava plantlets and their growth in the field. a) plantlets in culture room; b) plantlets planted in polybag covered with transparent plastic; c) the edge of plastic cover was cut after one week; d) all plastic covers were opened after 2 weeks; e) plants after one month in the green house; f) plants were moved to the field after 3 months in the green house.

Table 3. The surviving individual plants of 0.2-krad-irradiated Gebang regenerated on various MS medium supplemented with hormones after three months acclimatized in the green house.

Medium	Number of surviving plants
Ms 0	1.33 ± 1.53 ns
1/2Ms	0.0 ± 0.0 ns
MS+1 mg/L NAA+0.5 mg/L BAP	0.0 ± 0.0 ns
MS+1 mg/l BAP	7.22 ± 8.27 ns

Note: Means ± of standard deviation within column followed by the different letters in each column are significantly different at P≤0.05 by ANOVA.

Table 4. The number of surviving 0.2-krad-irradiated Gebang regenerated on various MS medium supplemented with hormones before and after acclimatization for three months in the green house.

Medium	Number of acclimatized plantlet	Number of surviving plants
Ms 0	22.67 ± 14.98 ns	1.33 ± 1.53 ns
1/2Ms	33.00 ± 24.04 ns	0.00 ± 0.00 ns
MS+1 mg/ L NAA+0.5 mg/L BAP	10.00 ± 2.65 *	0.00 ± 0.00 *
MS+1 mg/l BAP	53.89 ± 37.11*	7.22 ± 8.27 *

Note: Means ± of standard deviation followed by the superscript (*) showed significant differences between number of acclimatized plantlet and number of surviving plants at $P \leq 0.05$, respectively. Means ± of standard deviation followed by *ns* do not represent significant differences at $P \geq 0.05$.

The survival rate of stem cutting of 0.2 krad irradiated Gebang after three ratooning cycles tend to decrease after the second cycles of ratooning (Figure 4). It could be to the low quality of stem that affected to the numbers of survival stem cutting in the field. Therefore, it could be suggested that the cycle number of propagated stem plants related to the quality of growth and survival rate of plants. The more propagation cycle of stem plants using ratooning technique resulted less survival stem plants.

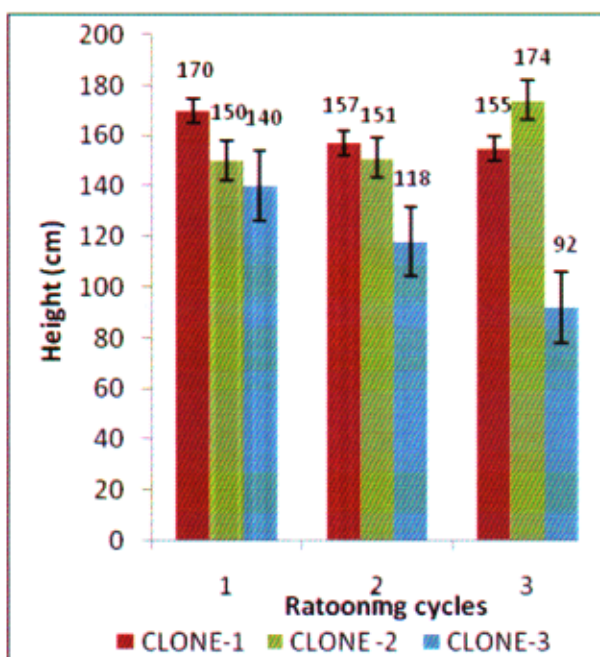


Figure 3. The height of three 0.2-krad-irradiated cassava plants (mother plant clones) prior to ratooning.

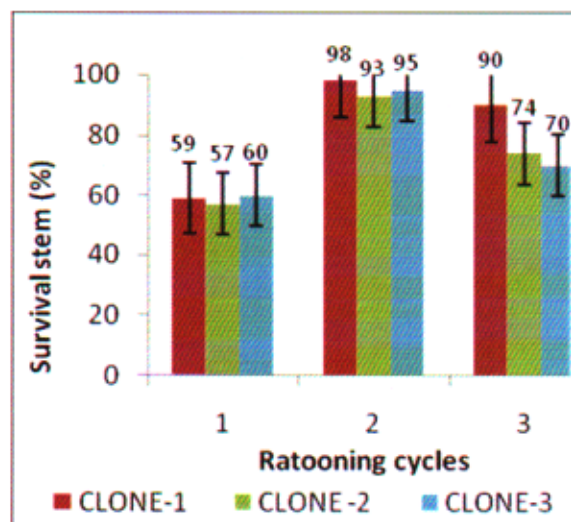


Figure 4. The survival rate of stem cutting of 0.2-krad-irradiated Gebang propagated from three mother plant clones after three ratooning cycles.

Conclusions

Most of 1.0, 1.5 and 2.0 krad gamma ray irradiated shoot cultures of Indonesian cassava Gebang genotype showed shoot phenotypic variations, while that of 0.2 krad showed normal appearances. Efficiency of plant regeneration and acclimatization relied on plant conditions such as the number of roots, leaves and the height of plantlets as well as media composition and humidity. The highest multiplication and acclimatization rate of irradiated shoot was obtained of 0.2 krad and multiplied in both MS medium and MS containing BAP at 1.0 mg/l. Subsequent multiplication of propagated stem increased in the second ratooning cycles and decreased especially in the survival rates afterwards. Incorporation of some of these favorable traits into breeding programs of Gebang will need further evaluation for the development of irradiated cassava in the field particularly concerning the yield and the alteration of genetic bases of irradiated cassava.

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