

Formation of upland rice drought-tolerant mutants by mutation induction and in vitro selection

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Abstract. Yunita R, Dewi IS, Lestari EG, Purnamanengsih R, Rahayu S, Mastur. 2020. Formation of upland rice drought-tolerant mutants by mutation induction and in vitro selection. *Biodiversitas* 21: 1476-1482. Increased production can be done by increasing the planting area, but the available land is sub-optimal land with drought stress. Drought-tolerant rice varieties are needed to utilize this land. To create drought-tolerant rice can use induction mutation and in vitro selection methods. This study aims to obtain tolerant upland rice mutants through mutation and in vitro selection. The parents used were Batutegi and Situpatenggang varieties. This research consisted of several main activities, namely callus induction, determination of LD 50% value of PEG determination of LC 50% value of gamma-ray irradiation, mutation induction and in vitro selection, bud regeneration and acclimatization. The results of this study are mutant callus formed by gamma-ray irradiation at a dose of 24.68 Gy for Situpatenggang and 22.15 Gy for Batutegi (LD50) and selected on PEG media at a dose of 24.11% for Situpatenggang and 25.18% for Batutegi (LC50). The mutant callus regenerated on MS + BA 3 mg/L + Zeatin 0.1 mg/L produced 83 Situpatenggang shoots and 73 Batutegi shoots and successfully acclimatized were 52 Situpatenggang lines and 49 Batutegi lines Batutegi

Keywords: Batutegi, gamma irradiation, LD 50% value of NaCl, *Oryza sativa*, Situpatenggang

INTRODUCTION

Rice (*Oryza sativa*) is very sensitive to drought stress. Lack of water will interfere with many cellular functions in plants and negatively affect plant growth and reproduction (Bray 2001). The response of rice plants to drought stress depends on the severity of the drought, growth phase (Kadir 2011), and genotypes (Castillo et al. 2006). Drought in the vegetative phase can inhibit leaf growth and roots, reduce the number of tillers and changes in root patterns (Mostajeran and Eichi 2009; Audebert et al. 2013), in the reproductive phase. Drought stress at panicle initiation will reduce panicle dry weight and number of grains per panicle, which has an impact on decreasing grain yield. This is caused by a decrease in photosynthesis, thereby reducing the production of assimilates for panicle growth and filling of grain (Akram et al. 2013). Drought stress not only suppresses growth and yield but also causes plant death (Djazuli 2010).

One way to overcome this problem is to use varieties that are tolerant to drought. Drought-tolerant plants are capable of adapting to drought conditions, which are shown by grain yields that do not decrease significantly (Haque et al. 1992). Drought-tolerant rice varieties are needed if dry season events are unpredictable, as is often the case lately (Pantuwan et al. 2002). Many techniques that can be used to assemble varieties are tolerant to drought, both

conventional and non-conventional. One technique that can be used to assemble drought-tolerant varieties is the method of mutation induction combined with in vitro selection.

Mutation induction method using gamma-ray irradiation is an effective method for increasing genetic diversity. Mutation induction techniques in combination with in vitro selection, can increase genetic diversity and increase the chances of obtaining new varieties that adapt to certain environments (Husni et al. 2006). Mutation induction techniques combined with in vitro selection have been shown to produce varieties that are tolerant of water shortages (drought), salinity, aluminum stress or pest and disease stresses. This technique is considered a breakthrough in promising plant breeding. This technique can accelerate the acquisition of new varieties with certain superior properties.

In vitro selection to obtain a genotype that is tolerant to drought can use selection agents in the form of osmotic compounds that can simulate dry conditions in the field. The osmotic compound that expresses the most dryness in the field is PEG. (Dami and Hughes 1997). This method has been tested on sugarcane (Rai et al. 2011; Mahmood et al. 2012; Hartati et al. 2018), Shorgum (Tsago et al. 2014), Rice (Wani et al. 2010; Joshi et al. 2011). The purpose of this study was to obtain plantlets of drought-tolerant upland rice mutants through mutations and in vitro selection.

MATERIALS AND METHODS

The plant material used was Situpatenggang and Batutegi rice varieties. This research consists of several activities, namely: callus induction, mutation induction by gamma-ray irradiation and in vitro callus selection, mutation induction and in vitro callus selection, callus regeneration, acclimatization of putative mutant plantlets.

Callus induction

This study uses a completely randomized design. The treatment was 2,4D concentrations (0, 1, 3 and 5 mg/L) with 10 replications. The explants used were zygotic embryos isolated from mature seeds. Sterilized seeds using sterile ingredients, including alcohol and chlorox. The embryos were then planted on callus induction media, namely, MS media contain 2,4-D (0, 1, 3 and 5) mg/L and Casein Hirolisate 3 g/L. In one bottle has ten explants and sterilized incubated on the culture shelf in the dark. The room temperature is 24 - 25°C. The variables observed were the percentage of embryonic callus formation, as well as a visual callus. Callus induction is performed repeatedly in order to obtain embryonic callus in large quantities.

Mutation induction by gamma-ray irradiation and callus in vitro selection

Determination of lethal dose 50 (LD₅₀) gamma-ray irradiation on callus

Callus were given irradiation treatment at concentrations 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 Gy, , each treatment consisted of 50 . The observed variable is the percentage of brown callus after four weeks. Data were analyzed to determine the lethal concentration. Determination of lethal concentration 50 (LC₅₀), using program CurveExpet 1.3.

Determination of PEG Lethal Concentration 50 (LC₅₀) on callus of rice

Callus induced from mature embryos were cultured in medium containing PEG that consists of multiple level concentrations of 0, 10, 20, 30, 40 and 50 mM, treatment consisted of 10 replicates, each replicates consisting 5 explants. Variables measured were the number of browning callus. Data were analyzed to determine the lethal concentration. Determination of lethal concentration 50 (LC₅₀), using the regression equation to find the equation of the best. The length of incubation callus in selection medium containing PEG is 4 weeks.

Formation of PEG-tolerant callus mutants using mutation induction by gamma-ray irradiation and in vitro callus selection

Callus formed was given irradiation treatment. The gamma-ray irradiation dose given is LD₅₀ Callus acclimatization which has been given mutation treatment is then transferred to the same medium for callus induction for four days. Callus that has been treated with mutations, transferred to callus proliferation media, added PEG as much as LC₅₀ values for each variety. Callus from the selection medium after two weeks was cultured on the same medium with the same PEG content for two weeks.

Selection is carried out for two periods and each period for two weeks. The variables observed are the number of browning callus.

Callus regeneration

This research uses a factorial completely randomized design. The first factor is BA concentration, the second factor is zeatin concentration. This research uses a factorial completely randomized design. The first factor is BA concentration, the second factor is zeatin concentration. The surviving callus on the selection medium was transferred to shoot regeneration media namely: MS contain BA (3, 5, 7 mg/L) and Zeatin (0, 0.1, 0.3 mg/L). The variables observed were percent regenerated callus forming whole plantlets in each rice variety used.

Plantlets acclimatization

This research uses a completely random design. The treatment is in vitro shoot varieties (Situpatenggang and Batutegi varieties). The resulting plantlets were acclimatized by means of the resulting plantlets being removed from the agar media and cultured on ordinary water media at room temperature. After the plantlets are able to form new roots, then plantlets are planted in mud media. The variables observed were percent of plantlets that lived in each rice variety.

RESULTS AND DISCUSSION

Callus induction

The 2,4d concentration treatment had a significant influence on the percentage of callus formed and embryonic callus in situpatengan and batutegi varieties. In the second week after planting zygotic embryo explants began to respond with swelling of the explants and began to form a callus. Explants cultured on media without 2,4-D were unable to form callus, different conditions for explants cultured on media containing 2,4-D could form callus (Table 1). Some research results indicate that 2,4-D is an effective auxin for initiating callus formation from zygotic embryos in various rice varieties (Bona et al. 2005; Tariq et al. 2008).

In Table 1, it can be seen that 2,4-D 3 mg/L of Situpatenggang and Batutegi varieties can induce 94% of callus, 70% of them are embryogenic in Situpatenggang varieties and 72% for Batutegi varieties. Increasing 2,4-D concentration to 5 mg/L can reduce the ability of explants to form callus and the percentage of embryogenic callus also decreases. Thus the concentration of 2,4-D 3 mg/L is the optimum concentration for forming callus in explants of upland rice embryos of Batutegi varieties and Situpatenggang. The same thing also happened in Fatmawati varieties where the best media for induction of embryogenic callus was enriched media with 2.4D 3 mg/L (Lestari and Yunita 2008).

The rice callus of the Situpatenggang variety and the Batutegi produced on the medium containing 2,4-D 3 mg/L were embryogenic in which the callus was yellowish-white, nodular and crumb (easily separated).

Table 1. Effect of 2,4-D concentration on callus formation in some upland rice varieties aged 8 weeks after planting

Concentration of 2,4-D (Mg/L)	Situpatenggang		Batutegi	
	Callus formation (%)	Embriogenic callus (%)	Callus formation (%)	Embriogenic callus (%)
0	0	0	0	0
1	46	23	58	39
3	94	70	94	72
5	83	35	95	53

Mutation induction with gamma-ray irradiation and in vitro selection of callus

Determination of lethal dose 50 (LD₅₀) gamma-ray irradiation on callus

Irradiation treatments showed different percentages of browning callus. The higher the dose of gamma irradiation given, the higher the percentage of browning callus (Figure 1) which shows the characteristics of death in callus (Agisimanto et al. 2016). The callus that has been irradiated will experience browning because of the degradation of the enzyme indolacetol dehydrogenase

which plays a role in the biosynthesis of IAA needed to regenerate somatic cell populations.

Figures 1 and 2 show the percentage of dead callus in line with the increase in irradiation dose. For Situpatenggang variety rice and Batutegi, giving irradiation dose of 30 Gy causes death in callus above 50%. Increased radiation dose of less than 30 Gy callus death more than 70%.

The results of the best curve-fit analysis program to determine the lethal dose of 50 (LD₅₀), obtained the best model equation based on the number of dead callus is $Y = 3.49 + 2.2X - 0.01X^2$ for Situpatenggang varieties and $Y = 8.35 + 2.16 X - 0.00X^2$ for Batutegi. The lethal dose of 50% (LD₅₀) based on the percentage of dead callus until four weeks after irradiation obtained lethal value of LD₅₀ dose for Situpatenggang variety is 24.68 Gy while for Batutegi variety is 22.152 Gy. This is in line with the results of other studies that obtained different LD₅₀ values for several rice varieties in the callus phase, for Binnatoa varieties is 5.0 Gy and for varieties of Pokali, BR-16 and BR-26 is 4.0 Gy (Hossain and Alam 2001). While the LD₅₀ value of rice varieties Bastami is 50 Gy (Saleem et al. 2005).

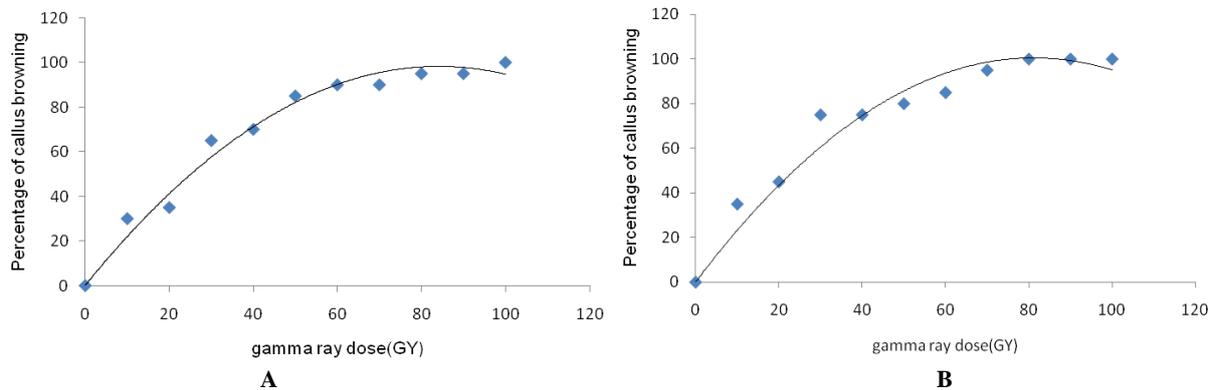


Figure 1. Percentage of browning callus, varieties of Situpatenggang (A) and Batutegi (B)

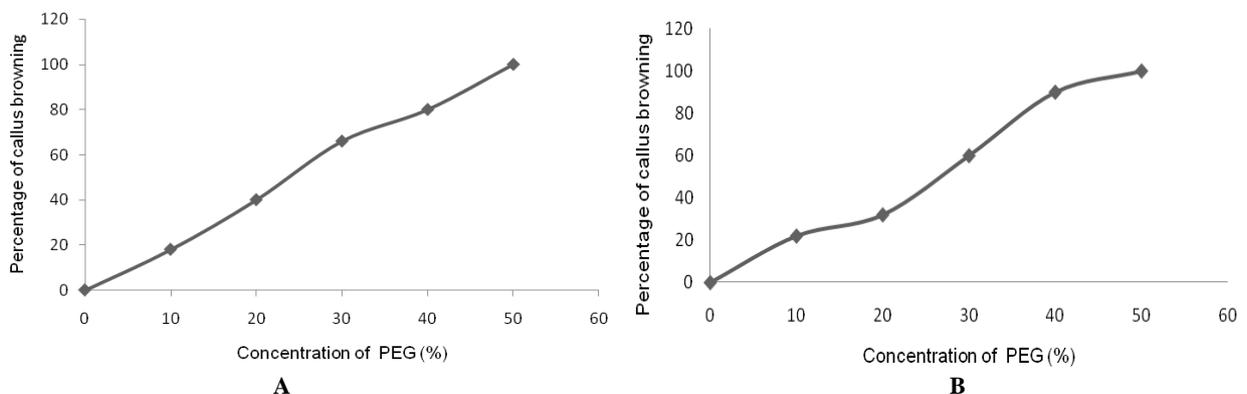


Figure 3. Percentage of callus browning on media containing PEG (A) Situpatenggang variety and (B) Batutegi

Determination of PEG lethal concentration 50 (LC50) on callus of rice

In Table 2, it can be observed that an increase in the concentration of PEG given will increase the number of browning callus. In Table 2 and Figures 3 and 4, it appears that an increase in PEG concentration results in an increase in the percentage of brown callus. For Situpatenggang and Batutegi rice callus, the 30 PEG treatment resulted in more than 40% brown callus. With increasing PEG concentration, it shows that somatic cells and plant tissue are not able to adjust to the increase in PEG concentration due to osmotic stress. Selected callus line grew better than non-selected callus when grown on different concentrations (Rao and Zabeen 2013).

The results of the best-fit curve analysis program to determine the lethal concentration of 50 (LC50), the best model equation obtained based on the number of brown callus can be seen in Table 3 and Figure 3 and 4. based on the LC50 PEG value, it can be seen the sensitivity level of varieties to PEG.

Table 3. shows that each variety has a different sensitivity to PEG. Batutegi variety has the highest LC50 value of 25.18% followed by 24.11% Situpatenggang. This shows that the Batutegi variety is slightly more tolerant of drought than Situpateng.

Formation of PEG-tolerant callus mutants using mutation induction by gamma-ray irradiation and in vitro callus selection

The embryogenic callus was cultured on MS media and then irradiated according to LD50. After being irradiated, the callus was transferred to the callus induction medium for recovery, restoring the callus and detecting contamination. Furthermore, the callus was selected on media containing PEG at LC50 concentration. After four days on callus selection media began to brown, the structure became soft. This shows the callus cells began to experience damage due to radiation stress and selection.

The callus responses of each variety to PEG varied. In the Situpatenggang variety, the percentage of brown callus was 61.66% and Batutegi was 61% (Table 4). Each variety has a different response to gamma-ray radiation (Yunita et al. 2014)

Selection of callus regeneration

In the 4th week after being planted on regeneration media, the callus starts to change. callus forms green spots even though the percentage is still small. Observation on the eighth week looks bud already appeared.

For the parameters of the number of shoots on callus varieties Situpatenggang, the treatment of BA 3 mg/L and Zeatin 0.1 mg/L gave the best response where the average number of shoots was 2.3. In the treatment of BA 3 mg/L and Zeatin 0.3 mg/L, there was a decrease in the number of adventitious shoots to 0.15 (Table 5). This shows that increasing the concentration of zeatin can reduce the number of shoots formed. The same thing happened in

cotton plant callus, increasing Zeatin content in growing media will reduce the ability of callus to form adventitious shoots (Lashari et al. 2008).

In the callus Batutegi variety, media containing BA 3 mg/L combined with Zeatin 0.1 mg/L were able to induce 95% shoot formation with a total number of shoots of 2.6. This treatment is the best treatment for shoot induction in rice varieties of Batutegi variety. Increasing BA to 5mg/L reduced the ability of callus to form shoots by 15% (Table 5). In this variety callus cultured on media without BA and Zeatin were unable to form adventitious shoots, because the callus requires cytokines to regenerate to form shoots (Ikeuchi et al. 2013).

From the above activities, the best medium for callus regeneration to form shoots in Situpatenggang and Batutegi varieties was MS Media which added BA 0.3 mg/L and Zeatin 0.1 mg/L. This formulation is used to regenerate calluses that have been mutated with gamma-ray irradiation at LD50 concentrations and selected on PEG media at LC50 concentrations.

In Table 6 it can be seen that from 115 callus Situpatenggang that were cultured on regeneration media, 83 callus which was able to form buds mean number of shoots was 1.2. In the Batutegi variety, of the 117 cultured calli, 73 calluses were able to form buds with an average number of 1.3 shoots (Figure 5).

Table 2. Percentage of callus browning on media containing PEG

Concentration of PEG (mM)	Variety	
	Situpatenggang (%)	Batutegi (%)
0	0	0
10	18	22
20	40	32
30	66	60
40	80	90
50	100	100

Table 3. The lethal concentration value of 50 (LC50) PEG of several upland rice varieties

Variety	LC50 value (%)
Situpatenggang	24.11
Batutegi	25.18

Table 4. In vitro selection of mutant callus on media containing PEG with LC50 concentration

Variety	Number of explants	Browning callus (%)	Callus performance
Situpatenggang	300	61.66	Callus browning
Batutegi	300	61	Callus browning

Table 5. Regeneration of callus forming shoots in Situpatenggang and Batutegei varieties

Concentration of zeatin (Mg/L)	Concentration of Ba (Mg/L)							
	0		3		5		7	
	Callus forms shoots (%)	Number of shoots	Callus forms shoots (%)	Number of shoots	Callus forms shoots (%)	Number of shoots	Callus forms shoots (%)	Number of shoots
Situpatenggang								
0	0	0	5	0.05	20	0.6	5	0.1
0.1	15	0.15	85	2.3	20	0.2	25	0.15
0.3	25	0.35	25	0.15	20	0.15	0	0
Batutegei								
0	0	0	10	0.1	25	0.5	10	0.2
0.1	10	0.15	95	2.6	15	0.15	25	0.35
0.3	35	0.65	20	0.3	15	0.25	10	0.15

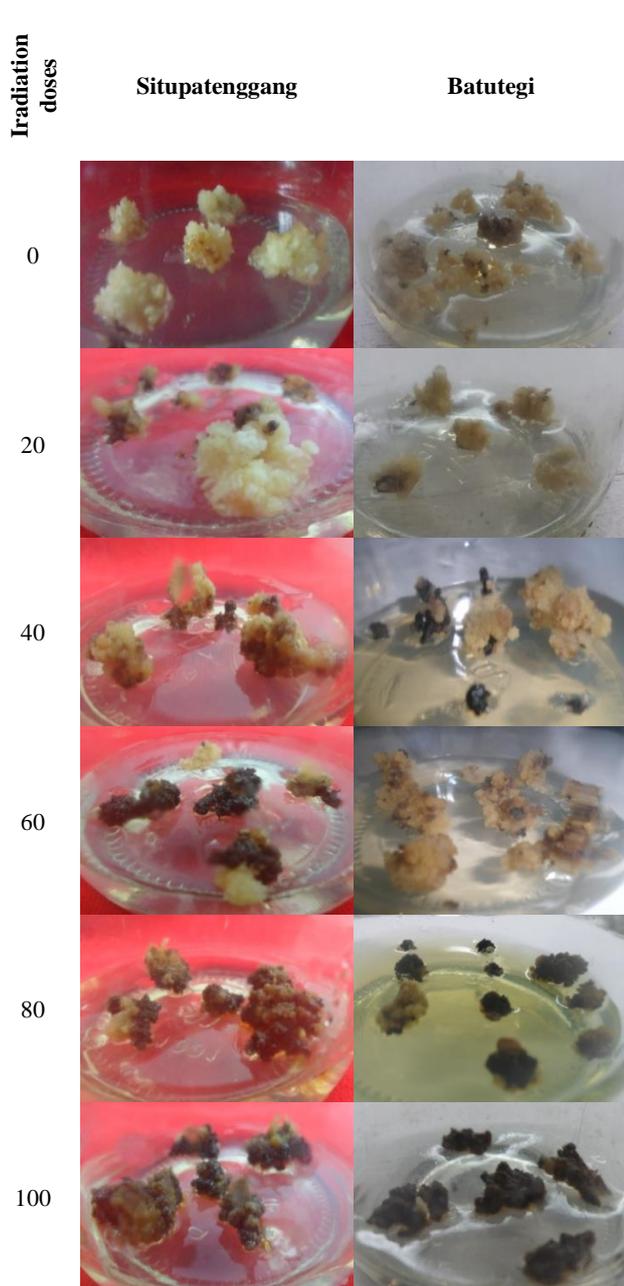
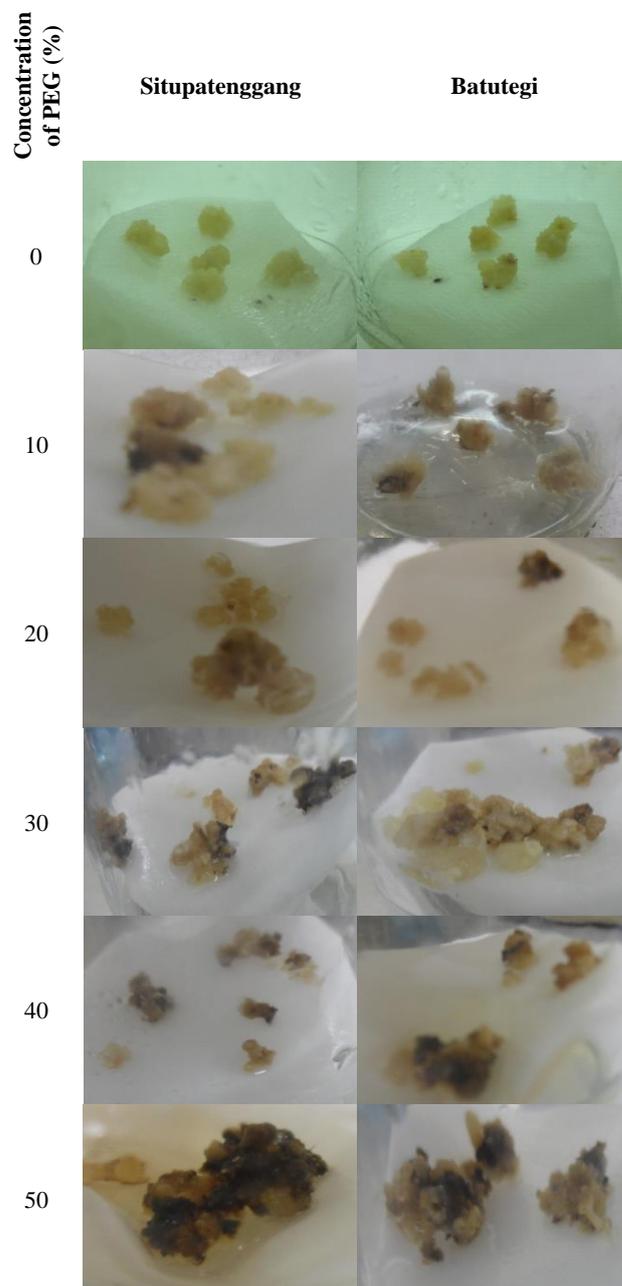
**Figure 3.** Rice callus performance Situpatenggang and Batutegei after irradiated gamma rays**Figure 4.** Performance of callus varieties of Situpatenggang and Batutegei after PEG treatment



Figure 5. Callus Situpatenggang regenerating to form shoots



Figure 6. acclimatization process of putative numbers of somaclon mutants

Table 6. Regeneration of selected callus

Varieties	Number of mutant calluses selected	Callus forms buds	The average number of shoots per callus
Situpatenggang	115	83	1,2
Batutegi	117	73	1,3

Table 7. Acclimatization of mutant plantlets

Variety	Number of plantlets	Plantlets remain green after acclimatization
Situpatenggang	70	52
Batutegi	70	49

Acclimatization

Acclimatization is an important stage, especially in plants resulting from *in vitro* culture, because in general, the roots of plantlets derived from *in vitro* culture have different structures so that the ability at the time of adaptation during acclimatization is not the same (Chandra et al. 2010). The percentage of successful acclimatization is usually very low, so the treatment and environmental conditions determine the success of acclimatization (Sharma 2017). To adjust to the environmental conditions acclimatization carried out in a way, plantlets were cultured on ion free for a week later after the plantlet was able to remove the new roots in which the plantlet was transferred to the mud media.

In Table 7, it can be observed that 70 plantlets derived from Situpatenggang variety rice have a number of living plantlets which is equal to 52. For Batutegi varieties, the number of plantlets acclimatized is 70 with the number of living plantlets being 49 plantlets (Table 7, Figure 6).

In conclusion, formation of upland rice mutants (drought-tolerant Situpatenggang and Batutegi varieties) was carried out by callus mutation with gamma rays at a dose of 24.68 Gy for Situpatenggang and 22.15 Gy for Batutegi (LD50) and selected on PEG media at a dose of 24.11% for Situpatenggang and 25.18% for Batutegi (LC50). The mutant callus was regenerated on MS + BA 3 mg/L + Zeatin 0.1 mg/L resulted in 83 Situpatenggang shoots and 73 Batutegi shoots. The shoots were acclimatized and produced 52 Situpatenggang lines and 49 Batutegi lines. The mutants produced need to be tested on land with drought stress. Selection is done in the vegetative and generative phases to get mutants that have higher yields than their parents.

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