Improvement of drought tolerance of patchouli through gamma irradiation and in vitro selection

S Suhesti¹, M Susilowati², N Sirait², W Haryudin² and E Hadipoentianti²

¹Indonesian Center for Estate Crops Research and Development (ICECRD), Indonesian Agency for Agricultural Research and Development, Jalan Tentara Pelajar No. 1, Bogor 16111, Indonesia
²Indonesian Spice and Medicinal Crops Research Institute (ISMCRI), Indonesian Agency for Agricultural Research and Development, Jalan Tentara Pelajar No 3, Bogor 16111, Indonesia

Abstract. The research was undertaken to obtain drought-tolerant patchouli putative mutant calli through gamma-irradiation and in-vitro selection using Polyethylene Glycol (PEG). Patchoulina 2 variety was used as the mother plant. Embryogenic calli were induced on three medium formulation (combination of 0.1; 0.3 and 0.5 mg/l 2.4D with 0.1 mg/l BAP). The induced embryogenic calli were then exposed to nine levels of Gamma irradiation (0, 5, 10, 15, 20, 25, 30, 35, and 40 Gray) in combination with two levels of PEG (0 and 20%). The results showed that MS media with 2.4-D 0.3 mg/l and BAP 0.1 mg/l was the best medium for callus induction. The ability of calli to survive decreased with increasing doses of gamma irradiation. The radiosensitivity of patchouli calli showed that LD₂₀ at 14.06 Gray and LD₅₀ at 26.98 Gray. The drought-tolerant patchouli putative mutant calli were obtained from gamma-ray irradiation doses 15, 20, and 25 Gray in selection media 20% PEG has better drought stress tolerance compared to parent Patchoulina 2 variety. Further selection in the glasshouse and field are needed to obtain a candidate variety of patchouli drought tolerance.

1. Introduction
Patchouli (Pogostemon cablin Benth.) is the basic raw material used in the pharmacy and essential oils industries in the world [1]. Patchouli oil is used as a fixative material in the production of perfume, soap, and cosmetics [2-4]. So far, the volatile substance of patchouli oil is unsubstituted [5]. Indonesia is the major producer that supplies more than sixty percent of the total world demand [6].

Patchouli cultivation in Indonesia is mostly on marginal lands dominated by dry land. Therefore, the plant is often experience drought stress that lower its production. Problems faced in patchouli development in dryland were the unavailability of drought-tolerant varieties. Currently, there are five superior patchouli varieties, in Indonesia namely, Tapak Tuan, Sidikalang, Lhokseumawe, Patchoulina 1, and Patchoulina 2 [6], but none of them were tolerance to drought stresses. Therefore, the development of patchouli drought-tolerant is needed to improve its production.

Patchouli is a non-flowering plant that propagates vegetatively due to its narrow genetic diversity of patchouli [2, 3, 6]. The combination of induced mutation and in vitro selection is regarded as a breakthrough in plant breeding. It has the potential to fast-track the development of new types with unique or superior characteristics. The most used induction mutation is physical mutation using gamma-ray irradiation [7]. Other researcher reported that around 3,362 mutant varieties had been successfully created through physical mutation technique [8]. Kinetic energy from gamma irradiation can change the
structure and position of genes or chromosomes. Thus, phenotypic changes occur and lead to new genetic diversity [9-10]. The induced mutation randomly occurred, so the combination with in vitro selection is needed to meet breeding objections.

In vitro selection has been acknowledged to expedite the production of somaclonal or mutant strains resistant to biotic and abiotic stressors. Drought-tolerant mutants can be created utilizing Polyethylene glycol in vitro selection (PEG). In addition, PEG can be used to simulate dryness in the field. [2,11-16].

The PEG compound is stable, non-ionic, water-soluble, and caused a decrease in homogenous water potential. This compound has a high molecular weight that caused water in media to be difficult to pass through the cell wall [16]. This situation can be used for simulation of groundwater potential decrease when drought stress occurs. To obtain the drought-tolerant patchouli plant, then formed putative mutant calli were subcultured in the media with an additional PEG selection agent. The survived calli assumed has a tolerant character to drought stress. Several study results have been done in drought stress tolerance includes for patchouli plant [16-17], sugarcane [18-19], and wheat [20]. This research objective was to obtain drought-tolerant patchouli putative mutant calli through irradiation with gamma-ray and in vitro selection using the selection agent Polyethylene Glycol (PEG).

2. Materials and Methods

The research was performed at the tissue culture laboratory of Superior Agricultural Seed Management Unit, Indonesian Center for Estate Crops Research and Development, Indonesian Agency for Agricultural Research and Development, Bogor, West Java, from March 2017 to September 2017. Patchoulina 2 variety was used in this research because of its character that is high productivity, resistant to bacterial wilt disease, and sensitive to drought stress. The study consisted of three steps, they were:

2.1. Induction of embryogenic callus

The explants were taken from the young leaf. The explant was sterilized with clean washing using detergent and soaked in the Dithane 1 gr/l solution for 30 minutes, Clorox 20% for 15 minutes, and alcohol 70% for 2 minutes. In every step, the explant was always rinsed with sterilized aquadest. The sterile explant was cut in 0.7 cm x 0.7 cm size and subcultured in media for callus induction. The media used basic media MS with additional growth regulator 2.4-D according to the treatment (0.1 mg/l; 0.3 mg/l; 0.5 mg/l) with 0.1 mg/l BAP cytokinin, 3 g/l sugar and 2.5 g/l phytagel compactor with pH 5.8. The culture was incubated at a temperature of 25°C in a dark condition. Subculture was done every 4 weeks in the same media. Calli from the best media were subcultured 3 times to obtain large quantities calli for mutation induction and in vitro selection.

This research used Complete Randomized Design with 3 treatment of callus induction media [3] that were: (1) MS + 0.1 mg/l 2.4-D + 0.1 mg/l BAP, (2) MS + 0.3 mg/l 2.4-D + 0.1 mg/l BAP, (3) MS + 0.5 mg/l 2.4-D + 0.1 mg/l BAP. Each treatment was consisting of 10 replications, each replication consisted of 5 bottles with 5 explants per bottle. The observed variable was time periods of calli initiation, percentage of explants that formed calli and the visual of calli.

2.2. Induced mutation through gamma irradiation

Induced mutation through gamma irradiation was conducted at the Irradiation and Radioisotope Application Center (BATAN), Jakarta. Gamma-ray irradiation was used to treat the embryogenic calli acquired in the preceding stage (Gamma Chamber 4000A with the active compound Co60). The research arrangement was a completely randomized design with nine doses of irradiation, i.e. 0, 5, 10, 15, 20, 25, 30, 35, and 40 Gray. Each treatment consisted of 10 replications, with 5 clumps of calli of each replication. Irradiated calli were immediately subcultured on the MS media without growth regulators (MS0) for two weeks, then subcultured into regeneration media 0.1 mg/l 2.4 D + 0.5 mg/l BAP [3] for two months. The sub-culture period was four weeks. They were incubated for 16 hours in a culture room with a light level of 1000 lux, and then was observed the percentage of survived calli The calli without symptoms of necrosis or browning were recorded in the percentage of survival calli. The Curve Expert Program 1.3 was used to analyze the data and determine the LD20 and LD50 levels.
2.3. In vitro selection for drought tolerance on gamma-irradiated mutants

In vitro selection to obtain drought-tolerant patchouli-mutant was carried out using PEG 6000. The patchouli calli irradiated with gamma-ray was immediately subcultured in the media without growth regulator (MS0) for 2 weeks recovery. Calli that well grown in the recovery media was then subcultured in the selection media MS0 with an additional PEG 6000 according to the treatment (0 dan 20%). The selection lasted for 4 weeks with subculture once in two weeks in the same media. The survived calli were then subcultured in the MS media with an additional 0.1 mg/l of 2.4-D and 0.5 mg/l of BAP [3]. Cultures were incubated under light exposure using TL lamps intensity of 1000 lux for 16 hours a day at a temperature of 20-22°C.

A two-factor Completely Randomized Design (CRD) was used to design the research. The first factor was gamma-ray irradiation doses (0, 5, 10, 15, 20, 25, 30, 35, and 40 Gray), while the second factor was the concentration of PEG 6000 (0 and 20%). Each treatment consists of 10 replications, and each replication has 5 clumps of calli. The treatment performed during the selection process to survival calli percentage, the relative decrease index (RDI) of calli in PEG was calculated using the following formula [21]:

\[
RDI = \frac{(V_c - V_s)}{V_s} \times 100\%
\]

where RDI = Relative decrease index, Vc = survival calli percentage on control media (PEG 0%), and Vs = survival calli percentage on selection media (PEG 20%). The data was then analysed its, variance using SAS 9.1 program followed by Duncan’s Multiple Range Test (DMRT) at a 5% level.

3. Results and discussion

3.1. Induction of embryogenic callus

At the beginning of the induction phase, the explant was swollen, curled and after that, the calli were formed especially around the wound. The swelling occurs because of the interaction between the explant and growth regulator through nutrition absorption. The media with the addition of 0.3 mg/l 2.4D and 0.1 mg/l BAP showed the best result compared with other treatments. Its percentage of calli formed was 75% with initiation times ranged from 13 to 18 days (Table 1).

| Treatment                  | Initiation Time (days) | % explant that formed calli | Visual of calli              |
|----------------------------|------------------------|-----------------------------|------------------------------|
| 0.1 mg/l 2.4-D + 0.1 mg/l BAP | 14-20                  | 62.5                        | Dry, friable, yellowish-white |
| 0.3 mg/l 2.4-D + 0.1 mg/l BAP | 13-18                  | 75                          | Dry, friable, yellowish-white |
| 0.5 mg/l 2.4-D + 0.1 mg/l BAP | 13-18                  | 70                          | Dry, friable, brownish-white |

Calli formed in the media with the addition of ZPT 2.4-D 0.5 mg/l formed calli with performance dry, friable and brownish-white. According to [22], calli with these characteristics is an embryogenic callus, and thus suitable mutation induction.

3.2. Induced mutation through gamma irradiation

The irradiation dose greatly affected the calli's ability to survive. The control treatment (0 Gray) had the highest percentage of survived calli, whereas 40 Gray, the maximum dose delivered, had the lowest ones (Tabel 2). The percentage of survived calli declined when the irradiation dose was increased. Thus, calli growth might be inhibited by increasing the gamma irradiation dose. This is in line with the research reported on sugarcane [23].
| Irradiation doses (gray) | Percentage of survival calli (%) | Calli Color        |
|-------------------------|---------------------------------|-------------------|
| 0                       | 95.90 a                         | Yellowish-white   |
| 5                       | 85.70 ab                        | Yellowish-white   |
| 10                      | 84.5 ab                         | Yellowish-white   |
| 15                      | 77.80 bc                        | Yellowish-white   |
| 20                      | 69.00 c                         | Yellowish-white   |
| 25                      | 44.20 d                         | Brownish-white    |
| 30                      | 42.20 d                         | Brownish-white    |
| 35                      | 38.40 d                         | Dark brown        |
| 40                      | 32.90 d                         | Dark brown        |

Notes: Numbers followed by the same letter in the same column were not significantly different at Duncan Multiple Range Test at 5% level.

According to [9], irradiation caused inhibition in cell multiplication and growth. Irradiation-induced plant cell death can occur both directly as DNA damage and indirectly as the toxic effect of free radicals such as H2O2 and OH- generated by water radiolysis. [24]. Calli damage will cause a decrease in regeneration ability and can even cause death and regeneration inability of the cell [25].

Gamma-ray irradiation treatment causes a change in the color of the calli. In comparison to the control, the calli did not change color at a lower dose (20 Gray). Calli with a yellowish-white color has a crumb structure and is more easily regenerated. In line with [17] statement, yellowish-white is a calli color with good quality and high regeneration ability.

Changes in callus color began to appear at 25 Gray, turning from white callus to brownish and go further to blackish-brown at doses above 25 Gray. The higher the dose used, the more blackish-brown callus color. Browning and blackening of callus were common in callus irradiated at high doses. This is probably due to the increase in the activity of the polyphenol oxidase enzyme at high irradiation doses. According to [26], Browning was caused by phenol oxidation following cell membrane degradation or cell disorganization. This process is an indicator of the formation of quinone as a result of the enzyme activity. This result is in line with the research of [23], which stated that irradiation at high doses could cause a change in callus color from yellowish-white to blackish brown, and callus tends to decrease its ability to regenerate. According to [17], callus with brownish or darkish color showed a decrease in the regenerate ability.

Various dosages of gamma-ray irradiation caused different levels of plant sensitivity (radiosensitivity). Radiosensitivity is measured to identify the best dose for producing the most mutants with the highest variability. The LD (lethal dose) is initiated the death of the irradiated plant population, hence was used as a determinant of radiosensitivity. A low radiation dose can cause the mutants to revert to their original forms (diploic selection), whereas a high radiation dose can result in sterility or death. Thus, most mutants are usually acquired around the lethal dose. [23].

The regression analysis showed that relationship between the calli survival rate and doses of irradiation followed a polynomial regression model, that is \( Y = 97.342 + 0.122 X - 0.127 X^2 + 0.002 X^3 \). Based on this equation, it can be determined that the 80% calli survival rate (LD20) is obtained at dose of 14.06 Gray while 50% survival rate (LD50) is obtained at a dose of 26.98 Gray. This indicated that the radiosensitivity (LD20-LD50) of patchouli calli variety Patchouлина 2 ranged from 14.06 Gray to 26.98 Gray. Mutant calli resulting from gamma-irradiation at doses between LD20 and LD50 are expected to produce high variability and thus provide a higher opportunity to obtain certain traits according to breeding purposes. This result is in line with research results reported by [17] and [27] which stated that patchouli calli treated with 20 Gray gamma-irradiation had the highest diversity and when exposed to 10-30 Gray was found to increase somaclonal diversity.
3.3. In vitro selection for drought tolerance on gamma irradiated mutants

Selection of mutant calli was conducted on MS medium containing 20% PEG for the duration of four weeks. The selection process ended when the elder of patchouli variety Patchoulina 2 (0 Gray/control) showed signs of damage calli with dark brown to black calli color. PEG concentration of 20% caused a change in callus color from yellowish-white into dark brown and black. These results are in line with the previous research conducted by [2] and [18], the calli grew on media with a concentration of 20% PEG was generally brown and black. According to [14], the color-changing of calli into brown indicates that the calli experienced water limitation due to the addition of PEG in the media.

According to [2], 20% PEG is a sub-lethal concentration of calli selection for Tapak Tuan variety with mortality percentage up to 90%. However, at 20% PEG, Patchoulina 2 variety showed a high percentage of live callus in all treatments. It indicated that the patchoulina 2 mutant was more tolerant to PEG stress conditions than that of Tapak Tuan.

The percentage of survival calli after drought selection using PEG was above 50% on average. The survived calli were indicates by healthy and yellowish-white in color, while the dead calli have changed color to dark brown or black (Figure 1 and 2). The yellowish-white calli indicate that mutant calli have passed in vitro drought selection at 20% PEG which was equivalent to -0.67 MPa [28].

The data analysis results in Table 3 showed a significant interaction between gamma-irradiation doses and PEG concentration to the percentage variable of survival calli. Therefore, the combination of the two treatment factors was analyzed as one factor. The results of two-factor interaction analysis showed that the highest percentage of survival callus was treated with 0 Gray irradiation dose cultured in 0% PEG media, while the lowest percentage of survival callus was in the 40 Gray irradiation dose mutant with 20% PEG media selection. This indicated that mutant callus with high irradiation dose (> LD50) experienced double stress due to high dose irradiation treatment and growth inhibition due to the addition of PEG in the selection medium. This causes the callus ability to live in the combination of treatments to decrease significantly, even this can lead to death so that the cells are unable to regenerate. Following the [25] statement, damage calli cause a decrease regeneration ability and even causes death so that cells were unable to regenerate.

Mutant calli irradiated with gamma-ray at each dose showed a significant decrease of viability on 20% PEG media selection compared to media without PEG (0%). This result showed PEG inhibits cell growth and development with binds water in media and disrupts the water availability for plant cells to grow and develop. Several studies had stated that this compound could reduce the water potential of the media as increase the genetic variation of plants [29]. This result is similar with those of [18-19 and 30], which showed that the addition of PEG as a selective media treatment inhibited the growth and development of cells or tissues.

In vitro selection using 20% PEG revealed that mutants generated from gamma-irradiation at 5 to 25 Gray doses had a larger percentage of survived calli than control parents (0 Gray). This result shows that the irradiation dose below the lethal dose of 50 (LD50) has a chance of obtaining drought-tolerant patchouli putative mutants. At 30 Gray and above, the calli had a significantly lower percentage of life calli than the elders. This result might cause the mutant calli from irradiation above the lethal dose (LD50) to experience doubled stress due to high-dose irradiation treatment that caused cell damage and growth inhibition by PEG that decrease the survived ability of calli. Following the [25] statement, damage calli cause a decrease regeneration ability and even causes death so that cells were unable to regenerate.
The decrease in the survival ability of calli to survive on the selection media could determine by the relative decrease index (RDI). The RDI value shows the level of tolerance of a living being to stress treatment compared to when it is unexposed to stress. The plant cell's ability to deal with stress showed by the RDI value. The smaller the RDI value, the higher the tolerance level to stressed treatment. Genotypes with a lower relative drought index were drought-tolerant varieties.

PEG is a drought selection agent, so callus that passes drought selection in vitro using a PEG selection agent with a lower RDI value expect to be more tolerant of drought stress in the field. The analysis results showed that patchouli callus resulting from gamma irradiation at doses of 30, 35, and 40 Gray showed a higher relative reduction index than the parental control (0 gray). This result showed that mutants from high irradiation doses above lethal doses of LD_{50} (>26.98 gray) experienced high cell damage caused mutants lost their ability to survive when exposed to drought stress in 20% PEG selection media. According to [25], the calli subjected to heavier selection pressures such as Gamma and PEG irradiation will experience physiological damage and metabolic disorders. (Table 3).

Patchouli calli resulting from gamma-ray irradiation at doses of 5 to 25 gray had a lower IPR value than the parent (control/0 gray) on 20% PEG selected media. This indicates that irradiation treatment at doses of 5 – 25 gray can produce better drought tolerance patchouli mutants than control parents (0 gray) on 20% PEG selection media (Table 3).

Table 3 shows that the lowest RDI value of patchouli calli found in mutants in the lethal dose range of LD_{20}-LD_{50} (14.06 – 26.98 gray), were in callus irradiated with gamma rays at doses of 15, 20, and 25 gray with RDI, respectively 18.25%, 14.41%, and 12.45%. This indicates that the radiation dose in the lethal dose range of LD_{20} – LD_{50} was the optimum dose to produce mutants according to breeding purposes. According to [24], the dose in the ranged of LD_{20}-LD_{50} is the highest dose to increase genetic diversity, thereby increasing the chances of breeders to obtain mutants according to the purpose of breeding. This is in line with [23] statement that the most suitable dose is usually around the lethal dose for producing most mutants.
Table 3. The effect of combining gamma irradiation and in vitro selection with PEG calli survival of Patchoulina 2 variety at 4 weeks.

| Irradiation dose (Gray) | Percentage of survival calli | Average | Relative decrease index (%) |
|-------------------------|------------------------------|---------|-----------------------------|
|                         | PEG concentration (%)        |         |                            |
|                         | 0                            | 20      |                             |
| 0                       | 92.50 a                      | 60.90 ghi | 76.70                       | 34.16          |
| 5                       | 88.30 ab                     | 69.60 efg | 78.95                       | 21.18          |
| 10                      | 87.30 abc                    | 66.30 fghi | 76.80                       | 24.05          |
| 15                      | 85.50 abc                    | 69.90 efg | 77.70                       | 18.25          |
| 20                      | 85.00 abc                    | 71.90 defg | 78.45                       | 15.41          |
| 25                      | 76.30 cdef                   | 66.80 fghi | 71.55                       | 12.45          |
| 30                      | 71.50 defg                   | 43.80 i  | 57.65                       | 38.74          |
| 35                      | 71.00 defg                   | 42.40 i  | 56.70                       | 40.28          |
| 40                      | 68.70 fghi                   | 38.40 j  | 53.55                       | 44.10          |
| Average                 | 80.68                        | 58.89 (+) |                             |

Notes: Numbers followed by the same letter in the same column were not significantly different at Duncan Multiple Range Test at 5% level. (+) indicated an interaction between irradiation level and PEG concentration.

Calli resulting from gamma-ray irradiation at doses of 5 and 10 Gray had an RDI value lower than the control (0 gray) but higher than RDI values of patchouli callus irradiated at doses of 15, 20, and 25 Gray. This showed that the 5 and 10 Gray mutants had better drought tolerance than the control (0 Gray), it was still far below the 15, 20, and 25 Gray mutants. Doses of 5 and 10 Gray are doses below LD 20 where the diploitic selection is possible to occur, its condition that mutant unstable and easy return to its original character. Increased drought tolerant and stable mutant calli obtained from irradiation doses of 15, 20, and 25 Gray. Mutants that passed in vitro selection using 20% PEG at doses of 15, 20, and 25 Gray expect better drought tolerance than their original parent Patchoulina 2. The mutant was then regenerated to form plantlets that would acclimatize in the greenhouse. Putative mutants that survive in drought selection in vitro will be evaluated further at greenhouse and field levels (Figure 3).

Figure 3. The regeneration of drought-tolerant patchouli putative mutant calli.

4. Conclusion
The best media for calli induction variety patchoulina 2 was MS media with 0.3 mg/l 2.4-D and 0.1 mg/l BAP. The ability of calli to survive decreased with increasing doses of gamma irradiation. The radiosensitivity of patchouli calli showed that LD20 at 14.06 Gray and LD50 at 26.98 Gray. The drought-tolerant putative mutant calli of patchouli obtained from gamma-ray irradiation dose 15, 20 and 25 Gray in selection media 20% PEG has better drought stress tolerance compared to parent Patchouli 2 variety. Further selection in the glasshouse and field are needed to obtain a candidate variety of patchouli drought tolerance.
Acknowledgment
We greatly appreciate the funding support from the State Government of 2018 fiscal year. We also thank Dra. Amalia, Lasia Seti Palindung, Totong Sugandi, Abdul Miftah, and Dewi Yuliyanti for the technical assistance in the laboratory and greenhouse.

Reference
[1] Huang H R, Wu W, Zhang J X, Wang L J, Yuan Y M and Ge X J 2016 A genetic delineation of Patchouli (Pogostemon cablin) revealed by specific-locus amplified fragment sequencing. J. Syst. Evol. 54 (5) 491–501. https://doi.org/10.1111/jse.12195
[2] Sutjahjo S H, Kadir A and Mariska I 2007 Effectiveness of polyethylene glycol as a selective agent on gamma irradiated patchouli calli for tolerance to drought. JIPI 9 (1) 48–57. https://docplayer.info/50261058-Efektivitas-polietilena-glikol-sebagai-bahan-penyeleksi-kalus-nilam-yang-diradiasi-sinar-gamma-untuk-toleransi-terhadap-cekaman-kekeringan.html
[3] Hadipoentjanti E, Amalia, Sirait N, Hartati S Y and Suhesti S 2008 Perakitan varietas untuk ketahanan nilam terhadap penyakit layu bakteri. Prosiding Konferensi Nasional Minyak Atsiri (Surabaya) pp 131–8. https://doi.org/10.21082/littri.v21n3.2015.131-138
[4] Wu L, Wu Y, Guo Q, Li S, Zhou K and Zhang J 2011 Comparison of genetic diversity in Pogostemon cablin from China revealed by RAPD, morphological and chemical analyses. J. Med. Plants Res. 5 (18) 4549–59. http://www.academicjournals.org/app/webroot/article/article1380728414_Wu%20et%20al.pdf
[5] Ramya H G, Palanimuthu V and Rachna S 2013 An introduction to patchouli (Pogostemon cablin Benth.) – A medicinal and aromatic plant: It’s importance to mankind. Agric. Eng. Int. CIGR J. 15(2) 243–50. https://cigjournal.org/index.php/Ejournal/article/view/2289
[6] Tahir M, Riniarti D, Ersan and Kusuma J 2019 Genetic and leaf characteristic diversity on 10 mutant progenies of patchouli (Pogostemon cablin) provide insights to selection strategies. Agrivita, J. Agric. Sci. 41(1) 139–48. https://doi.org/10.17503/agrivita.v41i1.1908
[7] IAEA 2020 FAO/IAEA Mutant Varieties Database. https://www.iaea.org/resources/databases/mutant-varieties-database
[8] Medina F I S, Amano E and Tano S 2005 Mutation Breeding Manual (Japan: Forum for Nuclear Cooperation in Asia) p 178. https://inis.iaea.org/search/searchsingerrecord.aspx?recordsFor=.SingleRecord&RN=37077897
[9] Ambavane A R, Sawardekar S V, Sawantdesai S A and Gokhale N B 2015 Studies on mutagenic effectiveness and efficiency of gamma rays and its effect on quantitative traits in finger millet (Eleusine coracana L. Gaertn). J. Radiat. Res. Appl. Sci. 8 120–5. https://doi.org/10.1016/j.jrras.2014.12.004
[10] Santos-Diaz M S and Ochoa-Alejo N 1994 PEG-tolerant cell clones of chili pepper: growth, osmotic potentials and solute accumulation. Plant Cell. Tissue Organ Cult. 37 1–8. https://doi.org/10.1007/bf00481110
[11] Dami I and Hughes H G 1997 Effects of PEG-induced water stress on in vitro hardening of ‘Valiant’ grape. Plant Cell. Tissue Organ Cult. 47 97–101. https://doi.org/10.1007/bf02318944
[12] Suprasanna P, Mirajkar S J, Patade Y V and Jain S M 2014 Induced mutagenesis for improving plant abiotic stress tolerance. Mutagenesis: exploring genetic diversity of crops ed Tomlekov M B, Kozgar M I and Wani M R (Wageningen Academics Publishing) pp 345–76. https://doi.org/10.3920/978-90-8686-796-7-17
[13] Saepudin A, Khumaida N, Sopandie D and Ardie S W 2017 In vitro selection of four soybean genotypes using PEG for drought tolerance. J. Agron. Indonesia. 45 (1) 14–22. https://doi.org/10.24831/jai.v45i1.13749
[14] Kaeem N S, Delporte F, Muhovski Y, Djekoua A and Watillon B 2017 In vitro screening of durum wheat against water-stress mediated through polyethylene glycol. J. Genet. Eng. Biotechnol.
[15] Hellal F A, El-Shabrawi H M, Abd El-Hady M, Khatab I A, El-Sayed S A A and Abdelly C 2018 Influence of PEG induced drought stress on molecular and biochemical constituents and seedling growth of Egyptian barley cultivars. *J. Genet. Eng. Biotechnol.* **16** 203–12. [https://doi.org/10.1016/j.jgeb.2017.10.009](https://doi.org/10.1016/j.jgeb.2017.10.009)

[16] Kadir A, Sutjahjo S H, Wattimena G A and Mariska I 2007 Pengaruh iradiasi sinar gamma pada pertumbuhan kalus dan keragaman planlet tanaman nilam. *J. Agro Biogen* **3**(1) 239–47. [https://doi.org/10.1016/j.jgeb.2017.04.004](https://doi.org/10.1016/j.jgeb.2017.04.004)

[17] Hartati R S, Suhesti S, Wulanndari S, Ardana I K and Yunita R 2021 In-vitro selection of sugarcane (*Saccharum officinarum* L.) putative mutant for drought stress in The 2nd International Conference on Sustainable Agriculture for Rural Development-2020 *IOP Conf. Series: Earth and Environmental Science Proceeding* **653** (IOP Publishing Ltd, Bristol, United Kingdom) p 12135. [https://doi.org/10.1088/1755-1315/653/1/012135](https://doi.org/10.1088/1755-1315/653/1/012135)

[18] Hartati R S, Suhesti S, Yunita R and Syafaruddin 2018 Induksi mutasi dengan kolkisin dan seleksi in vitro tebu toleran kekeringan menggunakan polyethylene glycol. *J. Penelit. Tanam. Ind.* **24**(2) 93-104. [https://doi.org/10.21082/littri.v24n2.2018.93-104](https://doi.org/10.21082/littri.v24n2.2018.93-104)

[19] Mahmood I, Razzaq A, Hafiz I A, Kaleem S, Khan A A, Qayyum A and Ahmad M 2012 In vitro selection of tissue culture induced somaclonal variants of wheat for drought tolerance. *J. Agric. Res.* **50**(2) 177–188. [https://apply.jar.punjab.gov.pk/upload/1374743993_95_546 165jarpap1(2).pdf](https://apply.jar.punjab.gov.pk/upload/1374743993_95_546 165jarpap1(2).pdf)

[20] Soeranto 2005 *Pemuliaan Tanaman dengan Teknik Mutasi* (Jakarta: Badan Tenaga Nuklir Nasional). [https://doi.org/10.17146/jair.2016.12.2.3225](https://doi.org/10.17146/jair.2016.12.2.3225)

[21] Biswas B, Chowdhury, Bhattacharya A and Mandal B 2002 In vitro screening for increased drought tolerance in rice. *Vitr. Cell. Dev. Biol.* **38** 525–30. [https://doi.org/10.1079/ivp2002342](https://doi.org/10.1079/ivp2002342)

[22] Laukkanen H, Rautiainen L, Taulavuori E and Hohtola A 2000 Changes in cellular structures and enzymatic activities during browning of Scots pine callus derived from mature buds. *Tree Physiol.* **20** 467–75. [https://doi.org/10.1093/treephys/20.7.467](https://doi.org/10.1093/treephys/20.7.467)

[23] Van Harten A M 1998 *Mutation Breeding: Theory and Practical Applications* (New York: Cambridge Univ. Press) p 353. [https://doi.org/10.2135/cropsci1999.0011183x003900030042x](https://doi.org/10.2135/cropsci1999.0011183x003900030042x)

[24] Mexal J, Fisher J T, Osteryoung J and Reid C P P 1975 Oxygen availability in polyethylene glycol solutions and its implications in plant-water relations. *Plant Physiol.* **55**(1) 20–4. [https://doi.org/10.1104/pp.55.1.20](https://doi.org/10.1104/pp.55.1.20)

[25] Maftuchah and Zainudin A 2015 In Vitro selection of jatropha curcas linn. hybrids using polyethylene glycol to obtain drought tolerance character. *Procedia Chem.* **14** 239–45. [https://doi.org/10.1016/j.proche.2015.03.034](https://doi.org/10.1016/j.proche.2015.03.034)

[26] Begum M K, Islam M O, Miah M A S, Hossain M A and Islam N 2011 Production of somaclone in vitro for drought stress tolerant plantlet selection in sugarcane (*Saccharum officinarum* L.). *Agric.* **9** (1&2) 18–28. [https://doi.org/10.3329/agric.v9i1-2.9475](https://doi.org/10.3329/agric.v9i1-2.9475)