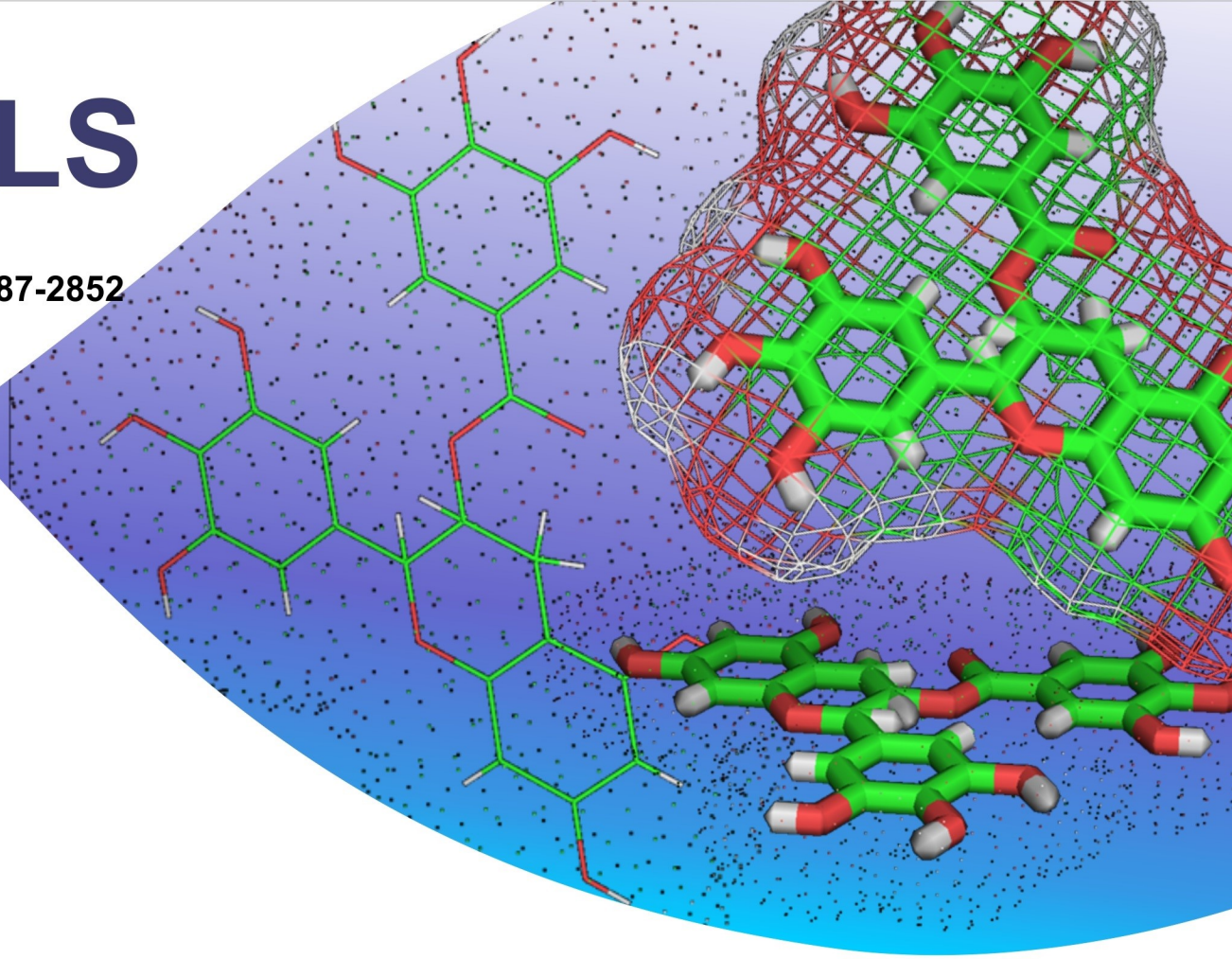
JELS

ISSN 2087-2852



The Journal of EXPERIMENTAL LIFE SCIENCE

J.Exp. Life Sci.	Vol. 10	No. 3	pages. 150-176	October 2020

Published by :
Graduate Program, Universitas Brawijaya

jels.ub.ac.id

ISSN. 2087-2852
E-ISSN. 2338-1655

The Journal of Experimental Life Science

Discovering Living System Concept through Nano, Molecular and Cellular Biology

Editorial Board

Address

The Journal of Experimental Life Science
Building B, 1st Floor, Postgraduate School, University of Brawijaya
Jl. Mayor Jenderal Haryono 169, Malang, 65145
Telp: (0341) 571260 ; Fax: (0341) 580801
Email: jels@ub.ac.id
Web: http://www.jels.ub.ac.id

ISSN. 2087-2852
E-ISSN. 2338-1655

Table of Content

The Effect of Ethyl Methane Sulfonate (EMS) on The In Vitro Shoot Regeneration of Vetiver (*Vetiveria zizanioides* [L.] Nash)

Wahyu Widoretno*, Serafinah Indriyani

Department of Biology, Faculty of Mathematics and Natural Sciences, University of Brawijaya, Malang

Abstract

Production of vetiver in Indonesia is low, and its oil quality is not in line with market demand due to the low quality of plant raw materials. *In vitro* mutagenesis using ethyl methanesulfonate (EMS) has the potential to produce superior vetiver variants. This study aimed to determine the effect of EMS on the growth and shoot formation of *in vitro* vetiver plants. *In vitro* mutagenesis was performed by culturing *in vitro* shoots on MS medium containing EMS with various concentrations (0.02; 0.04; 0.06; 0.08; 0.1%) for 4 weeks. The results showed that the addition of 0.02-0.1% EMS to the medium affected on *in vitro* shoot growth and the ability to form new shoots. EMS increased the percentage of dead explants and decreased the average number of shoots, the number of shoots formed per explant, and the shoot fresh weight. The higher the EMS concentration in the medium, the smaller the percentage of survived explants and the lower ability to form new shoots with the average number of shoots formed less. The percentage of survived explants on the medium without the addition of EMS and on the medium with the addition of EMS with low concentrations of 0.02 and 0.04% reached 100% with an average number of shoots formed ranging from 8.5-10 shoots/explant. Meanwhile, the percentage of survived explants on medium with the addition of EMS with a high concentration of 0.08-0.1% was only 12.5-25% with an average number of shoots formed was less than 2 shoots/explant.

Keywords: EMS, *in vitro* shoots, *Vetiveria zizanioides.*

INTRODUCTION

Vetiver oil derived from vetiver (*Vetiveria zizanioides* [L.] Nash) is one of the essential oils with a high market demand. It is used as a fixative in the perfume industry, a component mixture in the soap and cosmetics industry, and aromatherapy [1]. The use of vetiver oil in various industries is due to the presence of various chemical components of vetiver oil, namely vetiverol, vetivone, khusimone, khusimol, vetivene, khositone, terpenes, benzoic acid, triterpene-4-ol, β-humulene, epizizianal, vetivenyl vetivenate, iso khusimol, β-vetivone, and vetivazulene [2].

Vetiver production in Indonesia has not been able to meet the needs of vetiver oil because its production is low and it is not in accordance with market demand [3]. Its low quality is due to an accumulation of the low and non-uniform quality of essential plant raw materials as well as the less optimal refining process [4]. Therefore, to improve the quality of raw materials and oil yield, it is necessary to develop superior vetiver plant seeds.

In vitro mutagenesis, a combination of mutation induction and *in vitro* culture techniques is very effective for plant breeding programs because it accelerates the production of mutants. In addition to the high mutation frequency, *in vitro* mutagenesis also provides many choices of plant materials used for mutagen treatment such as *in vitro* axillary shoots, organs, tissues, protoplasts and cells which are more suitable for mutation induction techniques compared to *in vivo* shoots [5,6]. *In vitro* mutagenesis with EMS has been used successfully to obtain mutants in Saintpaulia (*African violet*) [7], Stevia (*Stevia rebaudiana*) [8], and cotton (*Gossypium hirsutum* L.) [9].

Besides producing variations in morphological characteristics, several studies showed that chemical mutagens EMS in high concentrations had an inhibitory effect on explant survival, callus formation, germination, shoot initiation and proliferation, root initiation, and plantlet regeneration [9,10,11]. Therefore, it is necessary to evaluate the effect of EMS on *in vitro* shoot regeneration in vetiver plants.

MATERIAL AND METHOD

Plant Material

Vetiveria zizanioides (L) Nash plants were collected from Sengklek, Pamalayan Village, Bayongbong District, Garut, West Java.

Multiplication of *In Vitro* Shoots

The source of explants used in this study was *in vitro* shoots obtained from crown explants. *In vitro* shoots were multiplied on MS medium with the addition of growth regulators NAA 0.1 mg.L^{-1} and BA 1 mg.L^{-1}. The culture was incubated at

* Correspondence address:
Wahyu Widoretno
Email : wahyu_widoretno@yahoo.com
Address : Dept. Biology, Brawijaya University, Malang 65145

ISSN. 2087-2852
E-ISSN. 2338-1655

24°C in light conditions for four weeks. The *in vitro* shoots of vetiver was used as material for *in vitro* mutagenesis using EMS.

Effect of EMS on Shoot Growth *In Vitro*

In vitro mutagenesis was performed using EMS. Small clumps of vetiver shoot (0.1 g) were cultured on MS medium + NAA 0.1 mg.L^{-1} + BA 1 mg.L^{-1} + EMS with several concentrations (0; 0.02; 0.04; 0.06; 0.08; 0.1%). Each mutagen treatment was repeated 10 times (10 bottles), with 5 clump shoots in each bottle. The cultures were incubated at 24°C in light conditions for 4 weeks. Each week, the growth of the culture was evaluated including the percentage of survived shoot explants, the percentage of explants forming shoots and the number of shoots formed. At the end of culture (4 weeks) the fresh weight of the shoots was measured.

Data analysis

The data were analyzed statistically using ANOVA (analysis of variance) and the differences among the mean values were compared with Duncan's Multiple Range Test (DMRT) ($P<0.005$) using ver. 25.

RESULT AND DISCUSSION

The addition of EMS in the medium affected the growth ability of *in vitro* shoots and the formation of new shoots (Fig. 1). In the medium without the addition of EMS, all *in vitro* shoots were able to grow and form new shoots, while the addition of EMS to the medium caused browning and death in some *in vitro* shoots and inhibited the formation of new shoots. The higher the EMS concentration in the medium, the more dead explants and the higher the inhibition of shoot growth in culture. Furthermore, the higher the EMS concentration in the medium, the less the ability of the explants to form new shoots.

All shoot explants cultured on MS medium with the addition of 0.1 mg.L^{-1} NAA growth regulator combined with 1 mg.L^{-1} BA without the addition of EMS survived and were able to form new shoots. The addition of EMS to the medium affected the percentage of survived explants, the average number of shoots, the number of shoots formed per explant and the fresh weight of shoots (Fig. 2).

The longer the culture period, the higher the percentage of dead explants, but the average number of shoots and the number of shoots formed per explant increased. At 4 weeks of culture, the percentage of survived explants on the medium without the addition of EMS and in the medium with the addition of 0.02% and 0.04% EMS was 100%, while the percentage of survived explants on the medium with the addition of higher EMS concentration, 0.06%, was 65%. The percentage of survived explants cultured on medium with a high EMS concentration, 0.08-0.1%, was only around 12.5-25% (Fig. 2A).

Besides affecting the percentage of survived explants, the addition of EMS to the medium inhibited the formation of new shoots. The fresh weight of shoot formed per explant on the control medium without the addition of EMS was 0.132 g, while the fresh weight of shoot in the medium with the addition of 0.02 and 0.04% EMS was 0.115 g and 0.081 g, respectively. Shoots formed on medium with higher EMS, 0.06-0.1%, had a fresh weight of 0.046-0.061 g or less than half the fresh weight of shoots formed on the control medium without the addition of EMS (Fig. 2B).

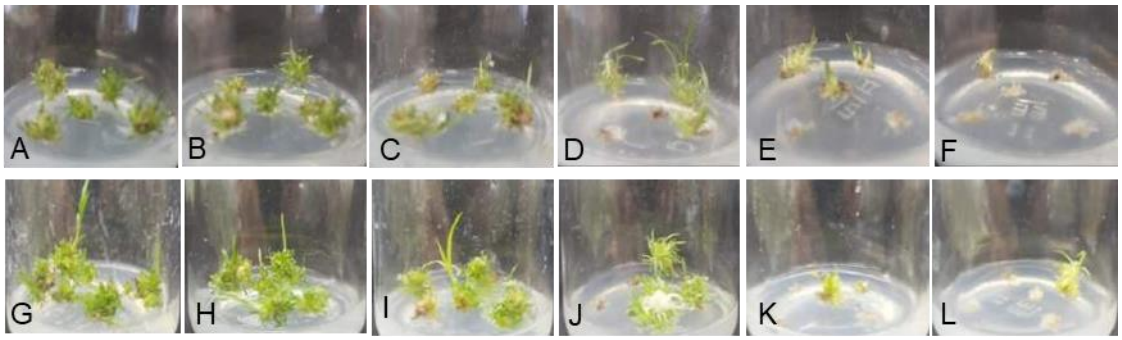


Figure 1. In vitro shoot explant growth response of *Vetiveria zizanioides* [L.] Nash to culture medium with the addition of EMS at 2 and 4 weeks of culture. A-F: 2 weeks, G-H: 4 weeks. A&G. without EMS (control), B&H. EMS 0.02%, C&I. EMS 0.04%, D&J. EMS 0.06%, E&K. EMS 0.08%, F&L. EMS 0.1%.

ISSN. 2087-2852
E-ISSN. 2338-1655

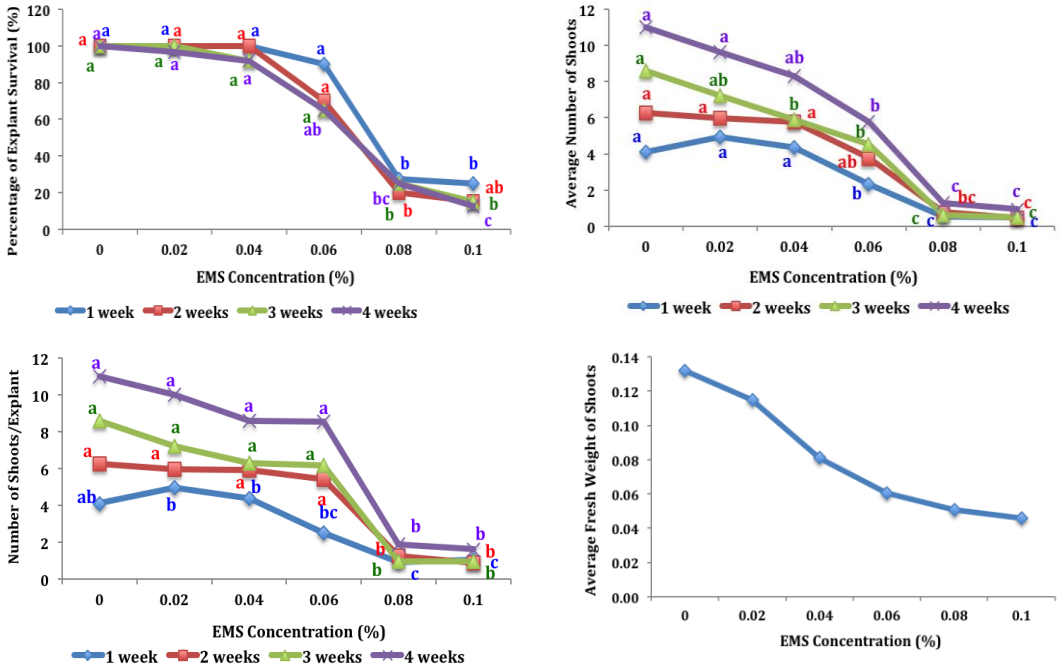


Figure 2. The effect of EMS on the medium on in vitro shoot growth of *Vetiveria zizanioides* [L.] Nash. A. Percentage of explant survival, B. Average number of shoots, C. Number of shoots/explant, D. Average of fresh weight/explant. Values followed by same letters for each culture period are not significantly different at 5% level as determined by Duncan's test (DMRT).

At the end of culture or 4 weeks after culture, the average number of shoots and the number of shoots formed per explant on the control media without the addition of the EMS mutagen was 11 shoots, while the average number of shoots and the number of shoots formed per explant on the medium with the addition of EMS 0, 02-0.06% ranged from 5.8-9.6 shoots (8.5-10 shoots/ explant). The average number of shoots and the number of shoots formed per explant on the medium with the addition of 0.08-0.1% EMS was between 0.9-1.3 shoots (1.6-1.9 shoots/explant) (Fig. 2C and 2D).

EMS mutagen added in the medium could reduce the viability of explants, growth and regeneration of vetiver shoots, and the higher the mutagen concentration, the higher the inhibition level of shoot regeneration. This suggests that the EMS mutagen in addition to inducing genetic variability is also toxic to *in vitro* shoot explants and causes disturbance toward some of the growth activities of the *in vitro* shoots of vetiver plants.

The effect of EMS on the inhibition of various growth parameters has been previously reported in some plants [8,11,12]. Immersion of 0.1-0.3% EMS for 30 minutes inhibited callus formation and shoot regeneration in Stevia [8]. EMS also reduced the percentage of survived explants, in vitro shoot regeneration and proliferation of chrysanthemums [11].

The frequency of shoot induction showed a decreasing trend with an increase in mutagen concentrations in citrus, stevia, and chrysanthemum [8,11,12]. There was a decrease in the number of regenerated shoots and shoot height and number of leaves with increasing concentrations of chemical mutagens [12,13].

Explant mortality cultured on medium with high EMS concentrations could be due to the toxic effect of mutagens [14]. Meanwhile, previous study mentioned that mutagens might cause disruption of hormone activity, especially cytokinins and physiological disturbances in shoot growth [11]. Physiological disturbances are mainly related to hormones and chromosome aberrations, which can cause inhibition of shoot initiation and proliferation and root induction.

CONCLUSION

The addition of EMS in the culture medium affect the ability of in vitro shoot growth and regeneration. EMS as mutagenic chemical reduce explants mortality and shoot regeneration, number of shoots formed per explant, shoots fresh weight and inhibit the formation of new

ISSN. 2087-2852
E-ISSN. 2338-1655

shoots. This suggests that addition of EMS mutagen not only induce genetic variability but also toxic to *in vitro* shoot explants and causes disruption of some of the growth activities of *in vitro* shoots of vetiver plants.

REFERENCES

[1] Inggrid, M., I. Levana, H. Djojosubroto. 2010. Perolehan dan karakteristik minyak akar wangi (*Vetiveria zizanioides*) hasil hidrodistilasi. *Seminar Rekayasa Kimia dan Proses*. Universitas Diponegoro. Semarang.

[2] Kumar, S.K., K.G. Gayathri, T. Kripa, Prathyusha. 2018. Preminilary phytochemical analysis and in vitro pharmacological evaluation of phytosterol rich fraction from *Vetiveria zizanoides* Nash. *Int. J. Res. Pharm.Sci.* 9(3). 922-930.

[3] Kardinan, A. 2005. Tanaman penghasil minyak atsiri komoditas wangi penuh potensi, 1st Ed. Agromedia Pustaka. Jakata.

[4] Mulyono. 2012. Peningkatan mutu dan efisiensi produksi minyak akar wangi melalui teknologi penyulingan dengan tekanan uap bertahap. *Buletin Teknologi Pascananen Pertanian.* 8(1). 35-47.

[5] Penna, S., S.B. Vitthal, P.V. Yadav. 2012. In vitro mutagenesis and selection in plant tissue cultures and their prospects for crop improvement. *Bioremediation, Biodiversity and Bioavailability*. Global Science Books. 6(1). 6-14.

[6] Predieri, S. 2001. Mutation induced and tissue culture in improving fruits. *Plant Cell Tiss. Organ Cult*. 64. 185-210.

[7] Fang, J.Y., S. Traore. 2011. In vitro mutation induction of saintpaulia using ethyl methanesulfonate. *Hortscience.* 46(7). 981–984.

[8] Gerami, M. H. Abbaspour, V., Ghasemiomran, H. Pirdashti. 2017. Effects of ethyl metahesulfonate on morphological and physiological traits of plant regenerated from stevia (*Stevia rebaudiana* Bertoni) Calli. *Appl. Ecol. Environ. Res*. 15(3). 373-385.

[9] Muthusamy, A. , J. Narayanasamy. 2011. In vitro induction of mutation in cotton (*Gossypium hirsutum* L.) and isolation of mutants with improved yield and fiber characters. *Acta Physiol. Plant*. 33(5). 1793-1801.

[10] Kona, P., H.M. Kumar, K.H.P. Reddy, D.M. Reddy, N.P.E. Reddy, P. Latha, M.S. Balaji. 2019. Effect of 2,4-D and EMS on in vitro regeneration in sugarcane cultivar, Co86032. *Intl. J. Curr. Microbiol. Appl. Sci.* 8(3). 1228-1236

[11] Padmadevi, K., M. Jawaharlal, 2011. Induction of in vitro mutation in chrysanthemum (*Dendranthema grandiflora* Tzvelev) Ray Florets (var. Ravi Kiran) using gamma rays and EMS. *Floriculture Ornamental Biotech*. 5(1). 74-77.

[12] Kaur, S. 2015. Effect of mutagens on regeneration and growt of in vitro grown epicotyl segments of rough lemon seedlings (*Citrus jambhiri* Lush). *J. Appl. Nat. Sci.* 7(1). 459-465

[13] Kashtwari, M., A.A. Wani, M.K. Dhar, S. Jan, A.N. Kamili. 2018. Development of an efficient in vitro mutagenesis protocol for genetic improvement of saffron (*Crocus sativus* L.). *Physiol. Mol. Biol. Plants.* 24(5). 951-962.

[14] Datta, SK., P. Misra, A.K.A Mandal. 2005. In vitro mutagenesis a quick method for establishment of solid mutant in chrysanthemum. *Curr. Sci.* 88(1). 155-158.

The Genetic and Morphoagronomy Character Diversity of Black Soybean Plant (*Glycine soja* (L.)): Responses to Mutation Induction by Gamma Rays Irradiation and Ethyl Methane Sulfonate

Shaddiqah Munawaroh Fauziah[*1] , Evika Sandi Savitri[2], Estri Laras Arumingtyas[3]

[1]Department of Biology, Facultyof Mathematics and Natural Sciences, University of Brawijaya, Malang, Indonesia
[2]UIN Maulana Malik Ibrahim Malang, Department of Biology, Faculty of Science and Technology, Malang, Indonesia
[3]Department of Biology, Faculty of Mathematics and Natural Sciences, University of Brawijaya, Malang, Indonesia

Abstract

Indonesia has a high dependence on imports of black soybeans. National black soybean needs continue to increase. Therefore, some efforts are needed to increase soybean production. Genetic resources of Black Soybeans with high diversity is also needed to develop superior varieties. One of the ways to increase soybean production is by mutation induction. Therefore, genetic resources of Black Soybeans with high diversity is needed to develop superior varieties. Mutagenesis using gamma rays and EMS can be used to increase genetic diversity. Doses used in this research were 1000 Gy gamma rays, 1% EMS, and combinations. This research was using a completely randomized design with morphoargonomy and molecular parameters. Morphoagronomy parameters observed were plant height, leaf area, seed weight, number of leaves, number of pods, and number of branches. ISSR markers with four primers (UBC 888, ISSR3, UBC876, and UBC889) were used to determine genetic variation of Black Soybeans. Based on the analysis of variance (ANOVA), it was shown that all parameters were able to give an effect on the morphoagronomy of black soybean plants. Gamma rays 1000 Gy were more effective in improving morphoagronomy of the plant in terms of plant height, seed weight, number of leaves, number of pods, leaf area, and number of branches. UBC 888 was the most effective primer to identify the genetic diversity of black soybeans that have been given mutation treatments.

Keywords: Black soybean, EMS, gamma rays, genetic variation, morphoagronomy.

INTRODUCTION

Black soybean (*Glycine soja*) is one of the members of the Leguminosae family [1]. The Ministry of Agriculture and related institutions in Indonesia have released several superior soybean varieties such as Detam, Cikurai, Malika, Mutiara, etc. This research was using Detam 3 variety because it was one of the superior types of a black soybean variety that is still rarely studied. Detam variety has *anthocyanin* levels of 222.49 mg.100g^{-1}, and *isoflavone* levels (genistein 0.65 mg.g^{-1} and daidzein 0.5 mg.g^{-1}) [2]. The lack of studies on this variety provides an opportunity for researchers to explore many unknown characters. Besides, Indonesia has a high dependence on imported beans, especially soybeans. In fact, Indonesia has a huge opportunity to increase legume production [3]. Based on data from the Central Statistics Agency (BPS), domestic soybean production was only 982.598 tons. Meanwhile, to meet the domestic needs, Indonesia has to import 2.6 million tons of black soybeans.

The main use of black soybean is for the raw material of soy sauce. At present, black soybeans are starting to be considered as raw materials for processing soybeans such as tempe and tofu because of their good content for people with diabetes mellitus [4]. Black soybeans contain *anthocyanins, isoflavones*, and *saponins* [5]. Those are antioxidants that play an important role in the regeneration of damaged cells. The more widespread use of black soybean has shown that it is necessary to increase production to meet domestic needs.

Due to low soy production at this time, it is needed to assemble superior varieties of soybeans. The availability of high diversity genetic resources is needed for this purpose. Mutagenesis is one of the ways to expand the genetic diversity of soybeans. Mutagenesis can be done using chemical and physical mutagens. One of the chemical compounds commonly used for mutagenesis is Ethyl Methane Sulfonate (EMS), while gamma rays are often used for physical mutations [6].

Research by Hanafiah on mutations in soybean varieties Agromulyo using 200 Gy treatment gamma-ray irradiation produced the highest diversity plants [7]. Gamma rays irradiation with the strength of 250 Gy was also gave an effect of increasing growth in almost all parameters of observation (shoot length, roots, fresh weight and leaf dry weight) to the *Canscora decurrens* plant [8].

* Correspondence address:
Shaddiqah Munawaroh Fauziah
Email : shaddiqah_2003@student.ub.ac.id
Address : Dept. Biology, University of Brawijaya, Veteran Malang, 65145.

ISSN. 2087-2852
E-ISSN. 2338-1655

EMS causes mutations at the DNA level by changing DNA bases (causing point mutations). It made slight damage to chromosomes, so it is beneficial in plant breeding activities [9]. Point mutations are also passed down to the next generation [10]. Whereas, soybean seeds soaked in 20 mM EMS for 10 hours produced soybean mutants with a total number of pods, contents, pod weight before drying, pod weight after drying, number of seeds, the weight of planting seeds, and weight of 100 seeds [11]. EMS mutagens can also be used at 0.05% to 2.5% concentration with 3 to 24 hours of soaking time [12,13].

Evaluation and characterization of mutant crops requires a long time and generally limited to morphological, anatomical, and biochemical characters. Therefore, it needs to do an efficient method that is not dependent on plant development [14]. Molecular characterization can be used to accelerate the process of crop selection. One of molecular the markers commonly used to detect polymorphism is Inter Simple Sequence Repeat (ISSR). ISSR is an effective technique in the genetic analysis of plant breeding programs. ISSR marker provides a quick method, low cost, and requirement for only small quantities of DNA [15], no need for DNA sequence information before amplification, high stability, and abundance of genomic information [16]. The percentage of Soybean's DNA polymorphism by using ISSR marker is about 70-100% [17].

Combination of gamma-ray irradiation and EMS as mutagen is still rarely applied to soybean plants. Previous research showed that EMS and gamma rays can increase the frequency of mutations Chlorophyll and sterile pollen in soybean MAUS-71 and JS-335 variety. [18]. Based on these facts, it was needed to observe the morphoagronomy character and genetic diversity in black soybeans induced by gamma-ray irradiation and EMS by using ISSR.

MATERIAL AND METHOD

Seeds of black soybean (Detam 3) were obtained from Research Institute for Various Nuts and Tubers (*Balai Penelitian Tanaman Aneka Kacang dan Umbi* - BALITKABI). This research was using a Completely Randomized Design (CRD).

Induction of Mutation by EMS

EMS 1% was dissolved it in Sodium phosphate buffer solution (pH 7) and DMSO 4%. The treatment was carried out at room temperature [19].

Induction of Mutation by Gamma Rays Irradiation

Black Soybeans were irradiated by 1000 Gy dose of gamma rays. We used CO-60 Gammacell 220 irradiator (dose 4585.5 Gy.hour^{-1}) at the National Nuclear Energy Agency of Indonesia (BATAN).

Table 1. Treatment Detail

Treatment Code	Treatments	Number of seeds
Control		
T0-without mutation	Seeds soaked in phosphate buffer pH 7 for 6 hours.	15
T1-without mutation	Seeds were planted without soaking.	15
Gamma rays irradiation		
T2-1000Gy	Seeds were irradiated by 1000 Gy of gamma rays.	100
EMS		
T3-1%	The seeds are soaked with pH 7 phosphate buffer for 6 hours, then soaked in EMS 1% for 6 hours.	100
Combination		
T4-1000Gy+1%	The seeds were irradiated with 1000 Gy gamma rays, then soaked in pH 7 phosphate buffer for 6 hours, and soaked in EMS 1% for 6 hours.	100

Planting Induced Mutations Seeds

The planting media used in this research was 3 kg of soil and sand with 1:1:1 of compost addition to each polybag. A total of 3 kg media was calculated from the weight of tillage per one hectare that is 2 million kg, and the optimum population of soybean per hectare that is 255.000 plants [20,21]. The media watered when the field capacity was 100% before use. A soybean seed planted in each polybag at ± 3 cm depth and carried out simultaneously until the harvest time.

Observation of Genetic Diversity

DNA Isolation, Purification and Quantification

The third or fourth apical leaf of black soybean was used for DNA isolation by using Doyle and Doyle protocol [22] with modification. DNA purity and concentration were determined using nanodrop (Nano 200-1002).

Electrophoresis

Electrophoresis was running at constant voltage 60 V for 60 minutes in 1x TBE buffer pH 8.0. The agarose gel used for DNA genome was 1% and 1.5% for PCR product. DNA ladder

ISSN. 2087-2852
E-ISSN. 2338-1655

(Promega) 100-1000 bp were used as the molecular size standards.

DNA Amplification by Using ISSR Marker

The components of the PCR reaction mixture and ISSR primers were used for this study can be seen in Table 2. The PCR reaction was performed using BioRad Thermal Cycler (Fig. 1).

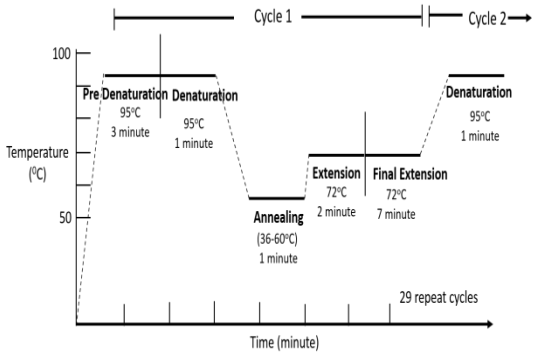


Figure 1. The PCR reaction

Morphoagronomy Character Observation

The morphoagronomy character observed were plant height (cm), seed weight (g), number of branches, number of pods, number of leaves, and leaf area. These characters were observed 30 days after harvest time.

Data Analysis

The data of morphoagronomy character of black soybeans were analyzed by using one way ANOVA then followed by DMRT test at 5% level to find out the best treatment of mutation induction. The ISSR product was scored for each primer then followed by the calculated percentage of polymorphism band. Statistical analysis was performed using the Paleontological Statistics Software Package for Education and Data Analysis (PAST). Dendogram was made via Un-weighted Pair Group Method with Arithmetic Average (UPGMA).

RESULT AND DISCUSSION

DNA Amplification

The result of DNA amplification using ISSR markers (Fig. 2) informs that there was a new DNA band appeared in the mutants, which where missing in the control. The different sizes of the DNA band indicated a change in the nitrogenous base as the result of mutation. When there is a mutation of DNA, the primer that initially attached to this site could not recognize it. The primer either did not attached to the initial site or attached to the new site formed by mutation. The amplification of this new site resulted in a new band with different sizes and thicknesses, so it made a polymorphism.

There were 45 fragments amplified from four primers. The number of fragments produced from each primer varies between 9-14 fragments. The result of polymorphic percentage ranged from 58.33-92.86%. ISSR3 produced the lowest polymorphic percentage (58.33%), and UBC888 has the highest value (92.86%) (Table 2).

The highest value of PIC, EMR and MI were generated by UBC888, the lowest value of PIC and MI were indicated by ISSR3, and the lowest values of EMR and RP were resulted by UBC889 (Table 2). PIC Values for dominant markers were between 0.0-0.5. The higher the PIC value, the better was the primer in determining genetic variation [22]. The EMR value is used to determine the number of polymorphic fragments in the observed sample. The higher EMR value showed the more effective primer in produce polymorphic fragments [23]. The MI value is used to determine the primer index in producing polymorphic fragments [24]. The RP value is used to determine the strength of a primer in producing a clear fragment. The higher the value of Rp, the better a primer in producing clear fragments [25].

Phylogenetic analysis

The similarity between Detam 3 and three other mutant varieties was based on the result of primer amplification. Phylogenetic analysis between accessions using PAST software were presented in a dendogram expressed by similarity value (Fig. 3). This result was calculated from three replication of each treatment except the control.

Tabel 2. ISSR primer amplification, % polymorphism of mutant soybean through gamma rays and EMS induction

No	primer		Ta (°C)	TNB	NPB	PB(%)	PIC	EMR	MI	RP
1	UBC889	5”-DBDACACACACACACAC-3”	53.0	9	6	66.67	0.45	4	1.80	11.82
2	ISSR3	5”-GTGTGTGTGTGTGTAC-3”	50.0	12	7	58.33	0.41	4.08	1.67	17.09
3	UBC876	5”-GATAGATAGACAGACA-3”	52.0	10	8	80	0.43	6.4	2.73	13.82
4	UBC888	5”-BDBCACACACACACACA-3”	53.0	14	13	92.86	0.49	12.07	5.93	15.82

Notes: TNB: Total Number Band, NPB: Number Polimorphic Band, PB: Polimorphic Band, PIC: Polymorphic Information Content, EMC: Effective Multiple Ratio, MI: Marker Index, RP: Resolution Power

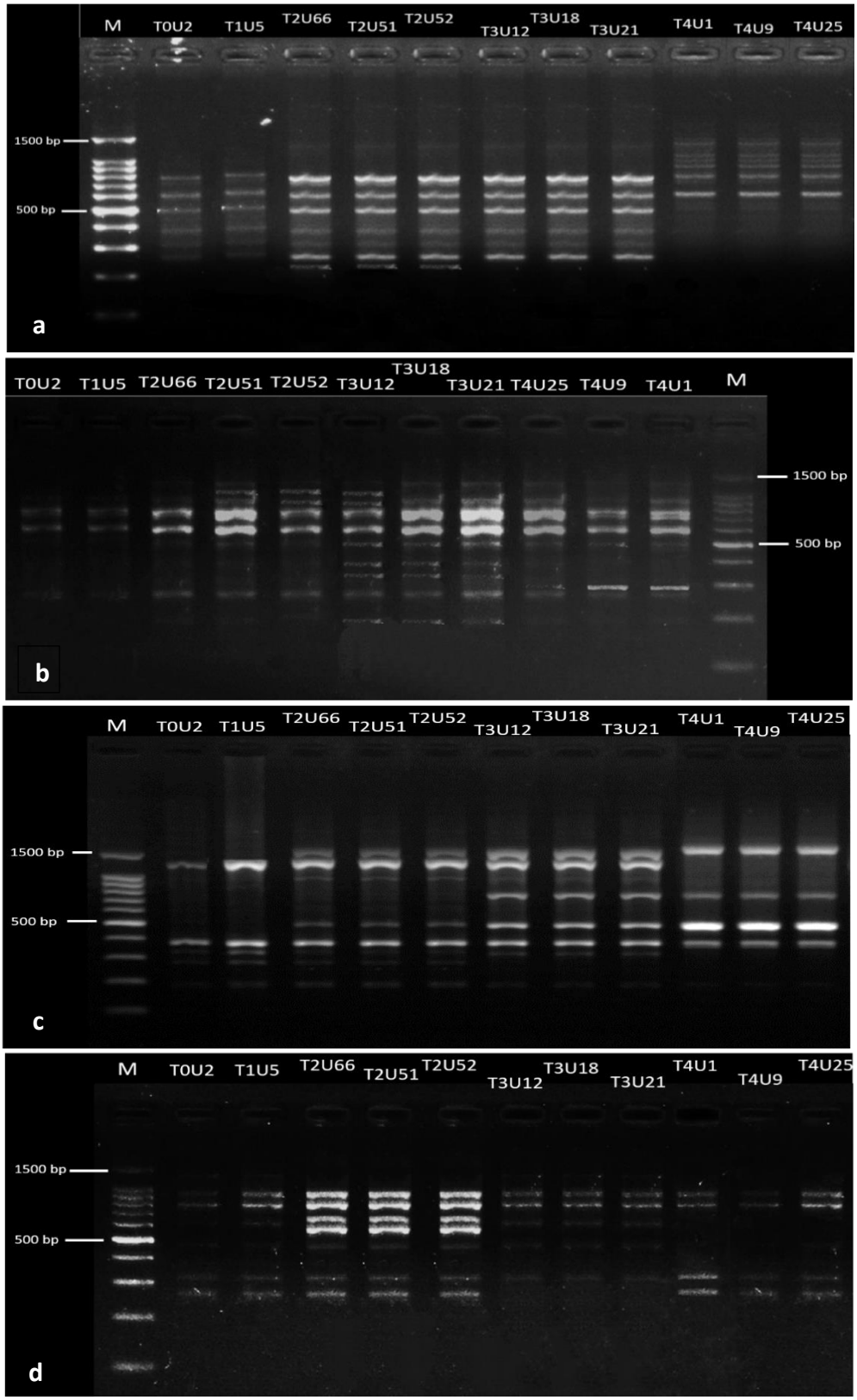


Figure 2. Profile of DNA amplification band for each primer (a) UBC 888, (b) ISSR3, (c) UBC876, (d) UBC889.
Description: *T= treatment, U=replication. T0= planted without mutation (Seeds soaked in phosphate buffer pH 7 for 6 hours), T1= planted without mutation (Seeds were planted without soaking), T2= 1000Gy, T3= 1% EMS, T4= 1000Gy+1% EMS.

ISSN. 2087-2852
E-ISSN. 2338-1655

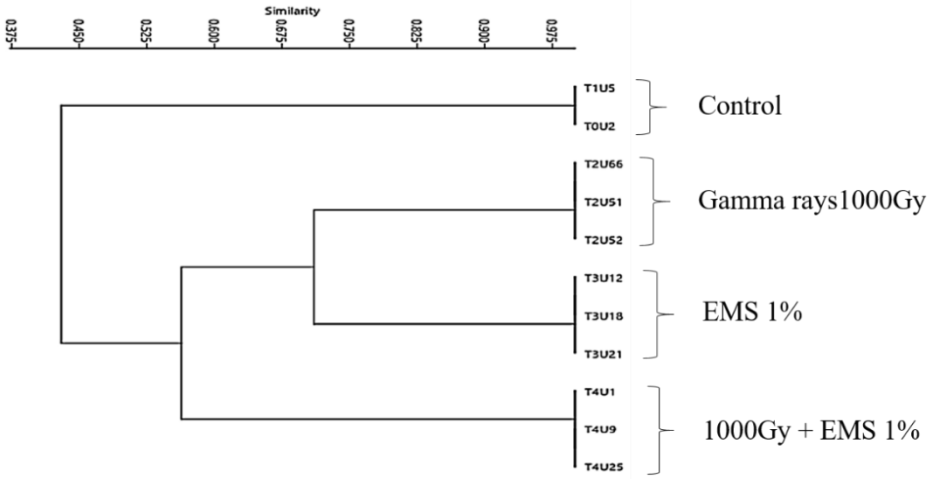


Figure 3. A dendogram of black soybeans (Detam 3) mutated by gamma rays and EMS
Description: *T= treatment, U=replication. T0= planted without mutation (Seeds soaked in phosphate buffer pH 7 for 6 hours), T1= planted without mutation (Seeds were planted without soaking), T2= 1000Gy, T3= 1% EMS, T4= 1000Gy+1% EMS.

The similarity of each treatment can be determined by the genetic coefficients. The genetic coefficient value was about 0 to 1. The closer value to 1 means the more similar genetically, and vice versa [26].

The dendogram in Figure 3 showed that between the control and others, there was a 0.425 similarity value. The sample of combination treatment has 0.55 in similarity with EMS and Gamma rays irradiation. And between the sample of gamma rays and EMS treatment has 0.69 similarity value. It showed that both EMS and gamma rays irradiation were effective methods to increase the genetic diversity of Black Soybeans.

Analysis of mutation treatment by EMS and Gamma rays irradiation showed changes in each treatment. It was caused by the change of DNA sequences so that the primer attachment site changed too. The research by Selvarasu showed that ISSR markers were able to show polymorphisms in *Gloria superba* plants induced by mutagens [27]. Aswandi also carried out research on the *Solanum lycopersicum* plant and reported that ISSR was a reliable primer for the initial and screening of mutagen [28].

Analysis of the effect of gamma ray irradiation and EMS on black soybean morphoagronomy

Morphoagronomy parameters observed in this study were plant height, seed weight, number of leaves, number of pods, and number of branches. Based on the analysis of variance (ANOVA), it was shown that all parameters observed had an $F_{count} > F_{table}$ of 5% level, which means that there was an effect of gamma-ray and EMS treatment on the morphoagronomy of black soybean plants. The significantly different results were further tested by Duncan Multiple Range Test (DMRT) on a 5% level (Table 3).

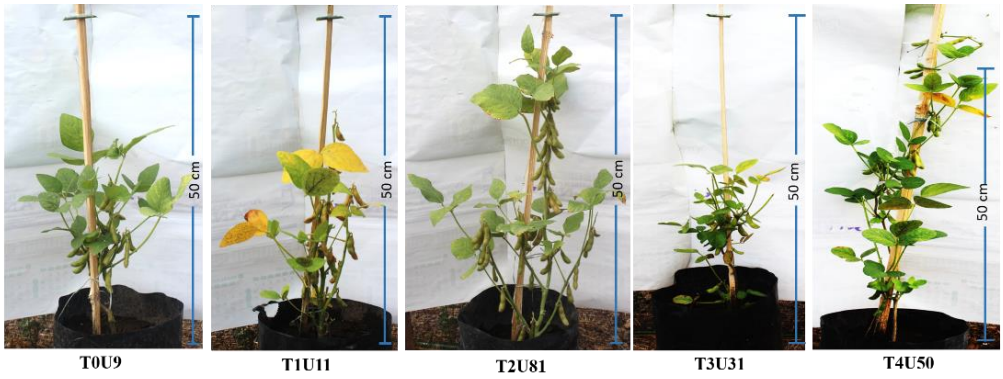


Figure 4. Habitus of Mutant Black Soybean Plants
Description: *T= treatment, U=replication. T0= planted without mutation (Seeds soaked in phosphate buffer pH 7 for 6 hours), T1= planted without mutation (Seeds were planted without soaking), T2= 1000Gy, T3= 1% EMS, T4= 1000Gy+1% EMS.

ISSN. 2087-2852
E-ISSN. 2338-1655

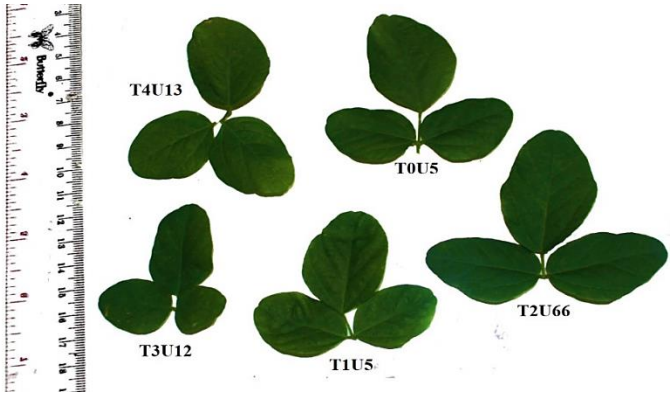


Figure 5. Leaf Area of Mutant Black Soybean Plants

Description: *T= treatment, U=replication. T0= planted without mutation (Seeds soaked in phosphate buffer pH 7 for 6 hours), T1= planted without mutation (Seeds were planted without soaking), T2= 1000Gy, T3= 1% EMS, T4= 1000Gy+1% EMS.

Tabel 3. Gamma rays irradiation and EMS effect to morphoagronomic character

Treatments	Plant height (cm)	Seed weight (g)	Number of pods	Number of leaves	Number of branches	Leaf Area ($cm^{2)}$
Gamma ray 1000Gy	46.71[c]	3.85[c]	24.87[c]	78.9000[d]	3.44[b]	**16.17[d]**
EMS 1%	24.69[a]	1.77[a]	13.06[a]	36.1923[a]	2.33[a]	10.54 [a]
Gamma ray + EMS	54.46[d]	3.30[b]	22.24[b]	67.3600[b]	2.40[a]	14.11[b]
Control (with buffer soaked)	41.79[b]	3.03[b]	20.53[b]	74.0000[c]	3.47[b]	15.15[c]
control (without buffer soaked)	41.70[b]	3.00[b]	20.53[b]	74.2000[c]	3.53[b]	15.00[c]

*numbers were followed by the same notation of DMRT (5%) means not significantly different

The plant height, leaf area, and the number of leaves of black soybean plants treated with gamma-ray irradiation and EMS were varied. The seed weight and the number of pods indicate that the treatment combination had the same effects as the control treatment. The number of branches in black soybeans treated with EMS and combination treatment tends to be the same. Table 3 showed that the EMS treatment caused a decrease in all quantitative variables. This result was in line with Wahyudhi, who reported that EMS could cause a decrease in the growth of Gogo rice seedlings [29].

Based on the result of this study, gamma rays tend to have a positive effect compared to other treatments. It has the same result with Meliala's research that gamma-ray treatment increased phenotypic diversity and plant height in upland rice [30]. Ragapadmi also reported that treatment with EMS has the ability to damage cells more than gamma-ray irradiation [31]. According to Astuti, gamma rays irradiation at certain doses can stimulate plant growth [32]. The growth was due to loss of meristem cell ability in devicied so that the cell activity was increased.

Gamma rays are short electronic waves with a high-energy level that can bind into atoms or molecules to produce free radicals in plant cells. Mutation induction using gamma rays irradiation produced the most mutants (around 75%) compared to other treatments such as chemical mutagens [33]. The diversity caused by mutagens was different in each species and even varieties. It can have both positive and negative effects depending on the concentration and duration of immersion given due to differences in genetic material and tolerance in responding to mutagen.

CONCLUSION

Gamma rays and EMS were able to produce genetic diversity in black soybeans Detam 3 varieties. UBC 888 was the most effective primer to identify the genetic diversity of black soybeans that have been given mutation treatments.

Gamma rays 1000 Gy were more effective in improving morphoagronomy.

The limitation of this research was the lack of mutagen dosage variation. For future study, it needs to use more doses of gamma rays and EMS. It is also a need to use more primers and specific genes to determine gene to determine the genetic diversity in mutated plants.

ACKNOWLEDGEMENT

The first author is highly acknowledging the supporting Department of Biology, Faculty of Science and Technology, UIN Maulana Malik Ibrahim Malang and Department of Biology, University of Brawijaya.

REFERENCES

[1] Johnson, H.W., R.L. Bernard. 1963. Soybean genetics and breeding. In: Norman, A.G. (Ed). The Soybean. Academic Press Inc. New York. 1-73.

[2] Nurrahman, M. Astuti, Suparmo, M.H.N.E. Soesatyo. 2012. Pertumbuhan jamur, sifat organoleptik dan aktivitas antioksidan tempe kedelai hitam yang diproduksi dengan berbagai jenis inokulum. *agriTech*. 32(1). 60-65.

[3] Fachruddin. 2000. Budidaya Kacang-kacangan. Kanisius. Yogyakarta.

[4] Zakaria, F.R., D.P.R. Firdaus, N.D. Yuliana. 2016. Konsumsi tahu kedelai hitam untuk memperbaiki nilai sgot/sgtp dan aktivitas antioksidan plasma penderita diabetes tipe 2. *Pangan*. 25(2). 95-104.

[5] Irwanto, R., D.R. Adawiyah, F.R. Zakaria. Peran fisiologis sari kedelai hitam diperkaya mikroenkapsulan minyak sawit mentah pada penderita diabetes melitus tipe 2. *Jurnal Teknololog*i *dan Industri Pangan*. 27(1). 1-9.

[6] Wiartana, I M.A. 2014. Induksi variasi cabai merah (*Capsicum annuum* L.) dengan ethyl methanesulfonate pada berbagai tingkat waktu perendaman. Master Thesis. Graduate School. Udayana University. Denpasar.

[7] Hanafiah, D.S., Trikoesoemaningtyas, S. Yahya, D. Wirnas. 2010. Induced mutation by gamma rays irradiation to Agromulyo Soybean (*Glycine max* [L] Merr.) variety. *Nus. Biosci.* 12. 105-111.

[8] Yadav, V. 2016. Effect of gamma radiation on various growth parameters and biomass of *Canscora decurrens* Dalz. *Int. J. Herb. Med.* 4(5). 109-115.

[9] Alcantara, T.P., P.W. Bosland, D.W. Smith. 1996. Ethyl methane sulfonate induced mutagenesis of *Capsicum annuum*. *J. Hered.* 239–241.

[10] Jabeen, N., B. Mirza. 2004. Ethyl methanesulphonate induces morphological mutations in *Capsicum annuum. Int. J. Agri. Biol*. 6(2). 340-345.

[11] Lukmaningtias, S.A. 2014. Pengaruh mutasi dengan *Ethyl Methane Sulfonate* (EMS) terhadap Pertumbuhan, Hasil dan kandungan karbohidrat tanaman kedelai (*Glycine max* L. Merrill). Thesis. Study Program of Agrotechnology, Faculty of Agriculture. University of Jember.

[12] Khan Z., H. Gupta, M.Y.K. Ansari, S. Chaudhary. 2009. Methyl methane-sulphonate induced chromosomal variations in a medicinal plant *Cichorium intybus* L. during microsporogenesis. Toxeminar-1. *Biology and Medicine.* 1(2). 66-69.

[13] Priyono, A.W. Susilo. 2002. Respon regenerasi in vitro eksplan sisik mikro kerk Lily (*Lilium longiflorum*) terhadap Ethyl methanesulfonate (EMS). *Jurnal Ilmu Dasar.* 3. 74-79.

[14] Wardiyati, T., E.L. Arumingtyas, M. Roviq. 2010. Evaluation of Scar18 marker linked to β-carotene for early screening of mango (*Mangifera indica* L.) progenies. *AGRIVITA.* 32(3). 261-270.

[15] Reddy, P.M., N. Sarla, E.A. Siddiq. 2002. Inter Simple Sequence Repeat (ISSR) polymorphism and its application in plant breeding. *Euphytica*. 128. 9-17.

[16] Wahyuni S., D.H. Xu, N. Bermawie, H. Tsunematsu, T. Ban. 2004. Skrining ISSR primer studi pendahuluan kekerabatan antar jahe merah, jahe empirit dan jahe besar. *Buletin TRO XV*. 1. 33-42.

[17] Monpara, J., K. Chudasama, V. Thaker. 2017. ISSR studies on small and large seed varieties of *Glycine max*. *J. Opharmacogn. Phytochem*. 6(6). 1652- 1656

[18] Satpute, R.A., R.V. Fultambkar. 2012. Effect of mutagenesis on germination, survival and pollen sterility in M1 generation of soybean (*Glycine max* (L.) Merrill). *Int. J. Recent Trends in Sci. Technol.* 2(3). 30-32.

[19] Narayanan, K.R., C.F. Konzak. 1969. Influence of chemical post-treatments on the mutagenic efficiency of alkylating agents. In: Induced Mutation in Plants. IAEA. Vienna. 281-301.

ISSN. 2087-2852
E-ISSN. 2338-1655

[20] Harsono, A., Sudaryono, B.S. Santoso. 2006. Maksimalisasi pemanfaatan radiasi matahari secara kultur teknis pada kedelai di lahan kering masam. Annual Report of Balitkabi 2006. Balitkabi. Malang.
[21] Irwan, A.W. 2006. Budidaya tanaman kedelai (*Glycine max* (L.) Merill). Padjajaran University. Bandung.
[22] Azmat, M.A.B., I.A. Khan, H.M.N. Cheema, I.A. Rajwana, A.S. Khan, A.A. Khan. 2012. Extraction of DNA suitable for PCR application from mature leaves of *Mangifera indica* L. *J. Zheijang Univ. Sci. B (Biomed & Biotechnol).* 213(4). 230-243.
[23] Roldan-Ruiz, I., J. Dendauw, E. van Bockstaele, A. Depicker, M. de Loose. 2000. AFLP markers reveal high polymorphic rates in ryegrasses (*Lolium* spp.). *Mol. Breed.* 6. 125-134.
[24] Varshney, R.K., K. Chabane, P.S. Hendre, R.K. Aggarwal, A. Graner. 2007. Comparative assessment of EST-SSR, EST-SNP and AFLP markers for evaluation of genetic diversity and conservation of genetic resources using wild, cultivated and elite barleys. *Plant Sci.* 173. 638-649.
[25] Prevost, A., M.J. Wilkinson. 1999. A new system of comparing PCR primers applied to ISSR fingerprinting of potato cultivars. *Theor. Appl. Genet*. 98. 107-112.
[26] Pratiwi, P. 2012. Analisis variasi genetik beberapa populasi *Globba leucantha* Miq. di Sumatera Barat dengan RAPD. Master Thesis. Sumatera. Andalas University.
[27] Selvarasu, A., R. Kandhasamy. 2017. Molecular and agro-morphological genetic diversity assessment of *Gloriosa superba* mutants. *Eur. J. Med. Plants.* 21(1). 1-13.
[28] Aswandy, A.B.K., N. Rawaidah, K.F. Rodrigues, C.B. Eng. 2015. Evaluation of the mutagenic effect of ethyl methanesulfonate on hexokinase nuclear DNA locus of *Solanum lycopersicum*. *Proceeding of Int'l Conf. on Advances in Science, Engg., Technology & Natural Resources (ICASETNR-15).*
[29] Wahyudhi A., T. Nurhidayah. 2014. The growth of M-1 seedling generation of the local varieties of upland rice (*Oryza sativa* L.) treated with mutagen Ethyl Methane Sulfonate (EMS). *Jurnal Faperta.* 1(2).
[30] Meliala, Jubleam Haris S., Nur Basuki dan Andy Seogianto. 2016. The effect of gamma irradiation on phenotypic changing in upland rice plants (*Oryza sativa* L.). *Jurnal Produksi Tanaman*. 4(7). 585-594
[31] Purnamaningsih, R., E.G. Lestari, M. Syukur, R. Yunita. 2011. Evaluasi keragaman galur mutan artemisia hasili radiasi gamma. *Jurnal Ilmiah Aplikasi Isotop dan Radiasi.* 6(2). 139-146.
[32] Astuti, D., Y. Sulistyowati, S. Nugroho. 2019. Radiosensitivity analysis of gamma ray to induce genetic diversity of high lignin content sorghum. *Jurnal Ilmiah Aplikasi Isotop dan Radiasi.* 15(1). 1-6.
[33] Kovacs E., A. Keresztes. 2002. Effect of gamma and UV-B/C radiation on plant cell. *Micron*. 33. 199-210.

ISSN. 2087-2852
E-ISSN. 2338-1655

The Effect of Methyltestosterone Hormone Immersion on Male Formation in Gourami Larvae (*Osphronemus goramy* Lacepède, 1801)

Tatang[1*], Maheno Sriwidodo[2], Harsuko Riniwati[2]

[1]Master Program of Aquaculture, Faculty of Fisheries and Marine Sciences, University of Brawijaya, Malang, Indonesia
[2]Faculty of Fisheries and Marine Sciences, University of Brawijaya, Malang, Indonesia

Abstract

The methyltestosterone hormone is a synthetic hormone of androgen and has been used to obtain the juveniles of male mono-sexual fish such as Tetra Congo, Tilapia, and Betta. The methyltestosterone use on gourami has not maximized. It because there is no data on the exact age of larvae to produce maximum male mono-sexual juveniles, so it is necessary to do research on the effect of methyltestosterone hormone on the age of gourami larvae on the success of male mono-sexual formation and get the right larval period to obtain maximum survival gourami. The methods of this research were conducted using a completely randomized design with four treatments of the age of gourami larvae aged ten days, 15 days, 20 days, and 25 days. Each procedure was repeated three times. Data analysis used ANOVA analysis and LSD test. The larvae are soaked in a solution containing 5 ppm of hormones for 24 hours. The results showed that the administration of the hormone methyltestosterone to gourami larvae (*Osphronemus goramy* Lacepède, 1801) with different ages had a very significant effect ($P < 0.01$) on male sex formation with the highest average percentage of males obtained in treatment B (15-day larvae age) that is equal to 82.33% and the lowest in the treatment of larval period of 10 and 20 days is 74.00%.

Keywords: Gourami, hormone, juvenile, methyltestosterone, *Osphronemus goramy*.

INTRODUCTION

The Giant Gourami (*Osphronemus goramy*) is an important commodity fish culture in Indonesia. They grow at a relatively slow rate, which can hinder efforts to increase production to meet high consumer demand. However, if increasing the production of this fish is difficult, it may be possible to increase the growth of each gourami fish. There are several known strategies to increasing the fish growth rate through the masculinization application.

Producing mono-sex juveniles means producing fish with one sex, only male or female. It is based on different fish growth patterns between male and female fish. In gourami, the growth of male fish is faster than female fish, males aged 10-12 months can reach an average weight of 250 g per head, whereas females only 200 g per head [1]. It means male growth is 20% faster than females. So by only producing male fish seeds can increase production from gourami enlargement efforts.

The primary approach to commercial applications of male populations is through hormonal and selective breeding that has produced a large interest. Sex reversal, manual sexing, hybridization, and super male production are different methods available to obtain the desired sex population. The hormonal sex reversal has been used as a valuable key tool in sex manipulation for aquaculture. There are several reports on the use of steroid hormone for successful induction of functional sex in ornamental fish that exist [2-5]. Sex reversal by immersion treatment with methyltestosterone (MT) is the most effective and practical method for the production of the all-male population.

The methyltestosterone hormone is a synthetic androgen hormone. This hormone has been widely used to obtain male mono-sex fish juveniles [6] and Louhan fish [7]. For gourami fish, methyltestosterone hormone with a dose of 5 $mg.L^{-1}$ can produce 66.98% of male gourami seeds [7]. However, these results have not been maximized. Unlike in Tilapia whose, the success rate can reach 90-100% male mono-sex. It is because data about the exact age of gouramy juveniles have not been obtained to produce maximum male mono-sex juveniles. This study aimed to evaluate the administration of methyltestosterone effect on the masculinization of juveniles of Gourami (*O. goramy*).

MATERIAL AND METHOD

This research was carried out in the Fish Reproduction Laboratory of State University of Malang. The study used a completely randomized design with four treatments of gourami larvae age (10 days, 15 days, 20 days, and 25 days) each procedure was repeated three times. Data analysis used ANOVA analysis and LSD test. One

* Correspondence address:
Tatang
Email : t4atank@gmail.com
Address : Faculty of Fisheries and Marine Sciences, University of Brawijaya, Veteran Malang, 65145.

ISSN. 2087-2852
E-ISSN. 2338-1655

hundred larvae per plastic bag were immersed in a solution containing five ppm of hormones for 24 hours for each treatment. The success of male-female formation was observed right after one month of morphological and histological maintenance [3], and survival of gourami fish was found at the end of the study.

RESULTS AND DISCUSSION

Percentage of Male Gourami

Giving methyltestosterone hormone at a dose of 5 ppm in the larvae of gourami fish aged 10, 15, 20, and 25 days after hatching by soaking for 24 hours. From the observations obtained the average data on the percentage of successful sex formation of gourami fish (*Osphronemus gourami* Lac.) presented in Table 1.

Table 1. Success Data of Sex Formation (%)

Age of juvenile	Male (%)	Female (%)
10-day	75.00	25.00
15 day	82.33	17.67
20 day	76.33	24.67
25 day	74.00	26.00

From Table 1, the results of the highest percentage of successful male sex formation were found in the treatment of 15-day-old larvae (82.33%). Meanwhile, the lowest portion of the successful male-sex structure was found in the treatment of 25-day-old fish larvae with the same value (74.00%) — the survival rate of gourami fish during one month of maintenance ranging from 74% to 82.33%.

The results of the variance above show that the administration of the methyltestosterone hormone gourami larvae (*Osphronemus gourami*) significantly affected the formation of male sex marked from the F count> F table 5%. So it continues on the Least Significant Difference test.

Tests of four different treatments for gourami larvae, the highest average percentage of males, were obtained in the treatment of 15-day fish larvae at 82.33%, and the lowest was at 25-day larvae treatments at 74.00%. It proves that with the age of 15 days, gourami fish larvae can direct changes in the direction of the higher male sex.

When the fish is 15 days old, the larvae have managed to direct the direction of their genitals into males by 82.33%. This means it is more effective and efficient when compared with previous studies [4], namely, the administration of the hormone methyltestosterone 5 $mg.L^{-1}$ to 21-day-old fish seed yields males 66.98% and requires soaking time of 3-6 hours [5]. The success of male sex formation when the fish is 15 days old of larvae is due to the right dose of hormones and is given at the correct phase, i.e., when the body is still smooth so that hormones are absorbed effectively through the skin by diffusion without being blocked by scales. Table 1 also shows that as the age of Gourami larvae increases, the percentage of males tends to decrease. It is because the bigger/older fish indicates that the fish has undergone a process of differentiation, fish that have differentiated channels and genitals have been formed so that the hormone methyltestosterone given will be challenging to influence sex changes. Besides, other factors that play a role in the process of sex formation are fish species, stocking densities, and environmental conditions.

Administration of steroid hormones is carried out when the gonad is still not formed (differentiated) and continues to be given until sex is fully differentiated, this will produce all males but 50% of the fish genotype produced by females [6]. The criteria for effective stimulation of sex reversal, namely steroid hormones are given when the gonad is still not formed and the treatment is carried out continuously until differentiated and the dose used must be sufficient and appropriate [7].

The effect of hormones on fish species has been studied. Methyltestosterone administration to *Tilapia zillii* after four weeks of age does not affect sexual changes and development [7]. Soaking eggs with eyespot and larvae of coho salmon in a methyltestosterone solution with a concentration of 25 $\mu g.L^{-1}$ was successful in changing the male sex to 100%. According to these results, it can be concluded that the treatment of hormonal doses must be appropriate and at the right age of the fish, and the dosage is specific to each fish species [8].

Survival Rate

The survival rate of gourami during one month of maintenance obtained data ranging from 70.76% - 80%. Data on survival in different larval ages can be seen in Figure 1.

During the maintenance period, the resulting life tends to be at a low rate. The low survival rate in this study is due to unstable temperatures wherein the morning it is too cold, and in the afternoon, the temperature is too hot, so the changing water conditions trigger the emergence of mold and make the fish condition often experience stress and die. This unstable temperature change took place from the beginning of the study up to 55 days during the

study. Therefore, the survival rate of gourami fish was low.

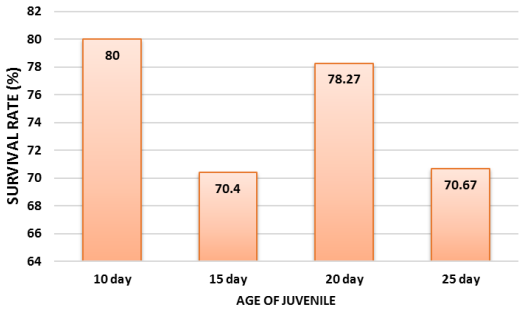


Figure 1. The survival rate of gourami during one month of maintenance.

Water Quality

For water quality during pond maintenance, the morning data was 22-25°C, afternoon for 27-30°C and evening at 24-25°C. Where the ideal temperature for support of gourami is 24-28°C [9]. Ranges of pH were 6.5-7.4 while the degree of acidity (pH) of water suitable for juvenile of gourami ranges from 6-8, and the dissolved oxygen ranges from 4-5 mg.L^{-1} while the best oxygen content for carp is between 4-6 mg.L^{-1} [9].

REFERENCES

[1] Pandian, T.J., K. Varadaraj. 1990. Techniques for produce 100% male Tilapia. *Naga, The ICLARM Quarterly.* 13(34). 3-5.

[2] Gale, W.L., Fitzpatrick M S, Lucero M, Contreras-Sanchez W Mand Schreck C B.1999. Masculinization of Nile tilapia (*Oreochromis niloticus*) by immersion in androgens. Aquaculture. 178. 349–57.

[3] George, T., T.J. Pandian. 1996. Hormonal induction of sex reversal and progeny testing in the zebra cichlid. Cichlasomanigro fasciatum. *J. Exp. Zool*. 275. 374–382.

[4] Pandey, G. 2013. Feed formulation and Feeding technology for fishes. *Int. Res. J. Pharm*. 4(3). 23-30.

[5] Zairin, M. Jr. 2000. Pengaruh pemberian bioenkapsulat 17α-Metiltestosteron di dalam artemia terhadap nisbah kelamin ikan cupang (*Betta splendens*). *Sains Akuantik.* 3. 1-8.

[6] Handajani, H. 2006. Pengujian hormon metiltestosteron terhadap keberhasilan pembentukan monosex jantan ikan gurami (*Osphronemus gouramy). Jurnal Protein* 13(1). 60-69.

[7] Aliu, B., J. Afolabi, M. Egwenomhe, A. Esume. 2017. Survey on nutrition and feeding practices of fish by farmers in Ondo State, Nigeria. *Asian J. Biol*. 2(1). 1–9.

[8] Handajani, H. 2006. Perendaman larva gourami (*Osphronemus gourami*) dengan umur yang berbeda pada hormon methyltestosterone terhadap keberhasilan pembentukan mono-sexual jantan. *Jurnal Protein*. 15(2).

[9] Handajani, H. 2006. Pengujian hormon methyltestosteronee terhadap keberhasilan pembentukan mono-sexual jantan ikan gourami (*Osphronemus gourami*). *Jurnal Protein*. 13(1). 60-69.

[10] Arifin, T.M., H. Handajani. 2005. Optimalisasi dosis hormone methyltestosteronee dan lama perendaman benih ikan gourami (*Osphronemus gourami* Lac.) terhadap keberhasilan kelamin jantan. Research Report. FPP-UMM. Malang.

[11] Zairin, M. Jr., Waskitaningtyas, K. Nasrum. Sumantadinata, 2001. Pengaruh Pemberian Artemia yang Direndam di dalam Larutan 17α-Methyltestosteronee Berdosis Rendah terhadap Nisbah Kelamin Ikan Cupang (*Betta splendens* Regan). *Aquaculture Indonesia.* 2. 107-112.

[12] Guerrero, R.D., W.L. Shelton. 1974. An aceto-carmine squash method for sexing juvenile fishes. *The Progressive Fish-Culturist*. 36(1). 56–56.

[13] Yamazaki, F. 1983. Sex differentiation. In: Hoar, W.S., D.J. Randal (Eds). Fish Physiology, Vol. III. Academic Press. New York. 117-158.

[14] Sitanggang, M., B. Sarowono. 2007. Budi daya gourami: Edisi Revisi. Penebar Swadaya. Jakarta.

Isolation and Screening of Phosphate and Potassium Solubilizing Endophytic Bacteria in Maize (*Zea mays* L.)

Hilyatul Azizah[1*], Saptini M. Rahajeng [2], Yoga D. Jatmiko[3]

[1]Master Program of Biology, Department of Biology, Faculty of Mathematics and Natural Sciences, University of Brawijaya, Malang, Indonesia
[2] Indonesian Center of Agricultural Training (ICAT) Ketindan, Lawang, Malang, Indonesia
[3]Department of Biology, Faculty of Mathematics and Natural Sciences, University of Brawijaya, Malang, Indonesia

Abstract

Maize (*Zea mays* L.) is a functional food source in Indonesia after rice and wheat. Increasing maize productivity can be done by developing a proper environment and nutritional availability during cultivation. The use of endophytic bacteria as biofertilizers is one prospective approach in reducing the synthetic fertilizers in the soil, and improving the absorption of macronutrients, such as nitrogen, phosphate, and potassium, as well. This study aimed to obtain phosphate solubilizing bacteria (PSB) and potassium solubilizing bacteria (KSB). The samples of maize leaves, stems, and roots were isolated using a serial dilution method and selected on a Pikovskaya (PKV) and Aleksandrov medium. A total of 10 PSB isolates were obtained from PKV selective agar media. The highest density was obtained in the root ($8.3x10^5$ $CFU.mL^{-1}$), stems ($5.7x10^5$ $CFU.mL^{-1}$), and leaves ($4.6x10^5$ $CFU.mL^{-1}$), respectively. Meanwhile, the diversity index of the PSB ranges from 0.7 to 0.78, indicates that the diversity index is high. Four isolates from PSB (AP1.3, AP3.1, AP1.2, and AP1.4) were selected as KSB because of the clear zone formed in Aleksandrov's medium. The highest production of phosphate and potassium was obtained by AP1.3 with value 12.11 $\mu g.mL^{-1}$ and 8.38 $\mu g.mL^{-1}$ at 72h and 15d, respectively. Therefore, these isolates potential to be used as an organic fertilizer (biofertilizers).

Keywords: Biofertilizer, endophytic bacteria, phosphate, potassium.

INTRODUCTION

Maize is the third staple food source after rice and wheat. According to the Trade Analysis and Development Agency (Badan Pengkajian dan Pengembangan Perdagangan - BPPP), Ministry of Trade, Republic of Indonesia the export value of maize in Indonesia in the 2010-2015 periods increased by 4.42%. Future agriculture is characterized by sustainable production processes that are able to remain productive without causing environmental impact [1].

Decreasing soil fertility due to depletion of soil organic matter and environmental damage requires a sustainable, agricultural system, namely organic farming. Through organic farming, it is expected, that it can maintain soil fertility, both physically, biologically, and chemically as in natural ecosystems by applying organic fertilizers, one of which is by utilizing the endophytic bacteria as agents of providing soil organic matter (biological fertilizers).

Biofertilizer is a fertilizer that contains live microorganisms. If these microorganisms are applied to seeds, plant surfaces, or soil, they promote their growth by increasing the supply of essential nutrients [2]. One of the important macronutrients is phosphorus and potassium which are required by plants to grow and develop. However, these efforts become efficient for plants because P is fixed by Al and Fe elements in acidic soils, while in alkaline soils, P will be fixed with Ca so that it was difficult for plants to utilize [3,4]. Meanwhile, the element of K is easily washed by water in the soil [5,6]. The ineffective usage of P and K fertilizers can be overcome by utilizing phosphate solubilizing bacteria (PSB) and potassium solubilizing bacteria (KSB) from plant tissues. Therefore, this study was to obtain phosphate solubilizing endophytic bacteria and potassium solubilizing bacteria from roots, stems, and leaves in maize (*Zea mays* L.).

MATERIAL AND METHOD

Sample Collection

The samples of roots, stems, and leaves of maize plants aged 45-60 DAP were taken from the maize plant conservation area of Indonesian Center of Agricultural Training (ICAT) Ketindan Lawang, Malang, Indonesia with the coordinates of the location (S 07049'53.2'') (E 112041'24.5'') and an altitude of 548 masl. Composite sampling was carried out at five points with three replicates.

* Correspondence address:
Hilyatul Azizah
Email : hilyaalawi@gmail.com
Address : Dept. Biology, University of Brawijaya, Veteran Malang, 65145

ISSN. 2087-2852
E-ISSN. 2338-1655

Isolation of Phosphate Solubilizing Bacteria

Surface sterilization was carried out by the method from Zhang *et al*., [7] with minor modification. Maize samples were cut (1-2 cm), a total of 15 grams of sample was washed with running water until clean, soaked in 70% ethanol for 2 min. Then soaked in 2% NaOCl solution for 3 min, and then rinsed with sterile distilled water four times (one minute each time). The sample (10 g) was homogenized with a sterile mortar and dissolved in 90 mL of 0.85% NaCl. Serial dilutions up to 10^{-6} was carried out, 0.1 mL of a sample from each dilution was inoculated on Pikovskaya agar (10 g glucose, 5 g $Ca_3(PO_4)_2$, 0.5 g $(NH_4)_2SO_4$, 0.2 g KCl, 0.1 g $MgSO_4$. $7H_2O$, 0.5 g yeast extract, 25 mg $MnSO_4$, 25 mg $FeSO_4$, and 20 g bacto agar in 1 L aquadest, pH ± 7) incubated for 3 d at 28°C [5]. Colonies that produce a clear-zone on Pikovskaya's agar medium were characterized to determine their diversity index.

The bacterial communities' diversity was determined by the Simpson diversity index [8]. Bacterial diversity is calculated from the number of bacterial isolates obtained.

$$D = 1 - \sum_{i=1}^{s} \frac{n_i(n_i - 1)}{N(N - 1)}$$

Description:
D = Simpson diversity index
ni = number of individuals of type i
N = total number of individuals
s = total number of species in the community

The PSB were selected on the basis of morphological parameters size, shape, colour, margin, and texture. All isolates were purified by quadrant streaking on nutrient agar slant for further testing.

Quantitative Assay of PSB

The ability of the isolated bacteria to solubilize phosphate was tested with a minor modification method from Lynn *et al.* [9]. One loop of the bacterial isolate was inoculated on Pikovskaya broth, incubated in a shaker at 120 rpm, 30°C, for 48 h. The suspension of bacterial culture (5 mL) with optical density 0.5 was inoculated in 45 mL of Pikovskaya broth. The cultures were incubated in a shaker at 120 rpm, 30°C for 96 h. The culture suspension (2 mL) was taken at incubation times of 0, 24, 48, 72, and 96 h and centrifuged at 10.000 rpm for 20 minutes.

The supernatant (1 mL) was added to 10 mL of chloromolibdic solution and 0.1 mL of chlorostannous acid, then added with sterile distilled water to a volume of 50 mL. The suspension was homogenated and incubated at room temperature for 10 minutes. After forming a blue color, the absorbance of the sample was measured at λ = 660 nm. The concentration of solubilize phosphate produced by each isolate was calculated based on the standard phosphate curve equation.

Potassium Solubilization Efficiency of PSB

The qualitative test was carried out by using the spot test method on Aleksandrov agar (5 g $C_6H_{12}O_6$, 0.5 g $MgSO_4.7H_2O$, 0.1 g $CaCO_3$, 0.006 g $FeCl_3$, 2 g $Ca_3(PO_4)_2$, 3 g $KAlSi_3O$ (K-feldspar), 20 g agar in 1 L of distilled water, pH ± 7). All the selected PSB strains were taken using a sterile toothpick, then, inoculated on Aleksandrov agar medium for 72 h at room temperature. After incubation, the diameter of the potassium solubilizing zone around the bacterial colonies was measured [5].

Quantitative Assay of KSB

The quantitative test of KSB was carried out based on a modified method [9,4,24]. Preparation of starter cultures was carried out as conducted in P solubilization. The culture (5 mL) was inoculated into 45 mL of Aleksandrov broth medium and incubated in a shaker for 15 d at 28° C. Every 5 d, 6 mL of the bacterial culture was centrifuged at 10,600 rpm, 28°C for 10 minutes. The supernatant was measured by using a Flame photometer. Standard curves were created using the KCl concentration.

Data analysis

The qualitative and quantitative data of phosphate and potassium solubilizing bacteria was analyzed based on a Two-Way ANOVA followed by a Tukey test using SPSS 16.

RESULT AND DISCUSSION

Diversity and Density of Phosphate Solubilizing Bacteria

A total of 10 endophytic bacterial isolates have been isolated from samples of roots, stems, and leaves of maize (*Zea mays* L.) on Pikovskaya agar (Fig. 1). The phosphate solubilizing bacteria density in the samples of roots, stems, and leaves of maize plants were $8.3x10^5$ $CFU.g^{-1}$, $5.7x10^5$ $CFU.g^{-1}$, and $4.6x10^5$ $CFU.g^{-1}$, respectively. The density calculation results showed the highest density was found in the root sample, followed by the stem and leaf. It is because roots are one of the main pathways for bacteria to enter plant tissues, and roots can easily absorb nutrients in the soil [10].

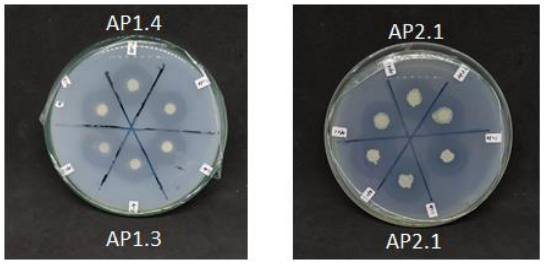


Figure 1. The clear zone formed on Pikovskaya selective media by PSB isolates with three replications.

The results of the Simpson diversity index calculation showed that, in both the solubilizing phosphate and potassium samples of roots, stems and leaves did not show any significant differences, with values ranging from 0.7-0.78. According to Ludwig and Reynolds [11], the closer to number one, the community between individuals is increasingly complex. Endophytic bacteria are bacteria that colonize plant tissue and are not parasitic during their life cycle. Several factors can influence the growth and development of endophytic bacteria, such as types of plants, environmental conditions (soil), community structure, and agricultural practices which also affect the colonization of endophytes in them [12].

Quantitative Estimation of PSB

Phosphate solubilizing bacteria (PSB) are soil bacteria that can dissolve phosphate so that it can be absorbed by plants. In addition to increasing phosphate in the soil, it can be improving plant root growth and increasing nutrient uptake. PSB is able to secrete organic acids so that it will reduce soil pH [13].

The results of the quantitative estimation of phosphate solubilizing endophytic bacteria showed that the isolate AP1.3 was able to dissolve the highest P at the 48 h, amounting to 10.34 µg.mL^{-1} and an increase at the 72 h to 12.07 µg.mL^{-1}. Meanwhile, the 96 h decreased by 8.97 µg.mL^{-1} (Fig. 2). Isolate BP1.3 dissolved the second-highest phosphate at 10.53 µg.mL^{-1} at 72 h and increased in concentration by 0.56 µg.mL^{-1} at 96 h. DP1.2, DP1.4, and DP2.2 from the 0 to 96 h incubations time did not show any significant increase in phosphate production with an average production value of 2.48-5.61 µg.mL^{-1}. It is because some PSB isolates require a longer incubation time than other isolates.

The previous study showed that the quantitative results of the PSB PEEHME5 isolate could dissolve 99 µg.mL^{-1} of phosphate within 7 d of incubation [12]. *Terribacillus saccharophilus* strain 002-048 was able to solubilize the highest phosphate of 0.29 µg.mL^{-1} at 48 h [14]. The difference in the ability to solubilize the phosphate is influenced by different types of bacteria. In addition, it is also because the gene expression of each isolate is different in secreting organic acids [15].

PSB is able to utilize phosphate sources in liquid media containing $Ca_3(PO_4)_2$ as a source of insoluble P. The phosphate dissolution mechanism in the liquid media occurs due to the release of insoluble P into soluble. Previous studies stated that several bacterial genera have been reported as PSB, namely *Azotobacter, Burkholderia, Citrobacter, Enterobacter, Pantoea*, and *Pseudomonas* [5,6].

Phosphates play a role in the process of decomposition of carbohydrates, transferring energy, forming nucleoproteins, root growth, accelerating maturity, producing fruit and seeds. Phosphorus plays an indispensable role as a universal fuel for all biochemical activities in the living cells [16,28]. The use of biological fertilizers (biofertilizers) in agricultural land is still limited by farmers in Indonesia. Agricultural Census of the Central Statistics Agency (BPS) in 2013 reported that 86.41% of farmers used inorganic fertilizers. Meanwhile, the use of balanced fertilizers (organic and inorganic) is only 13.5% and organic 0.07%.

Qualitative Estimation of KSB

The qualitative results of potassium solubilizing showed four PSB isolates were capable of producing clear zones on Aleksandrov media within 72 h (Fig. 3). The clear zone formed on selective media is due to the production of polysaccharides or enzyme activity [17].

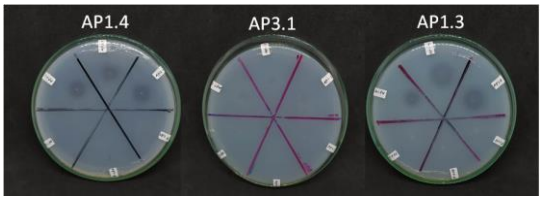


Figure 3. The clear zone formed on Aleksandrov selective media by PSB isolates with three replications.

The large clear zone formed on the media is not always caused by the size of a bacterial colony. In addition, the clear zone in the agar medium cannot represent the total concentration of solubilize phosphate or potassium. *Bacillus* sp. KF668 isolates could show clear zones at an incubation time of 48-96 h [12]. AKSB12, AKSB16, AKSB20, and AKSB24 isolates showed clear zones at 72 h [18].

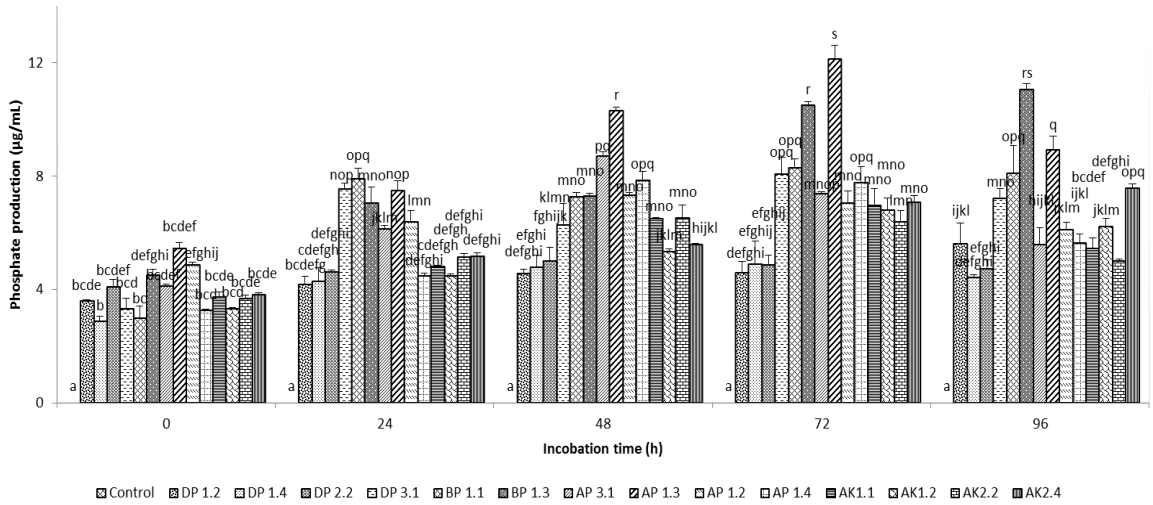


Figure 2. The quantitative test results of the phosphate solubilizing bacterial isolates at various incubation times. Data were expressed as mean ± standard deviation of the three replications and the notations on this histogram indicate differences between treatments ($p < 0.05$).

The Ability of Phosphate Solubilizing Bacteria to Solubilize Potassium

Potassium is a macronutrient essential for plant growth. Potassium is needed in plants for metabolisms such as cell synthesis, enzyme production activities, protein, cellulose, and vitamins. Also, potassium can increase plant resistance to abiotic and biotic stress [26]. Potassium (K) also plays a role in regulating the transport of water and nutrients through the xylem. A sufficient amount of potassium guarantees plant vigor and stimulates root growth and strengthens stems, which means increasing plant resistance to pathogenic fungal attacks [27].

The results of the quantitative estimation of potassium solubilizing endophytic bacteria (Fig. 4.) showed that the AP1.3 can dissolve the highest potassium at the incubation time 10 days 7.68 µg.mL^{-1} and has increased to 8.38 µg.mL^{-1} at 15 days. On the 15th day, the concentration of all isolates showed optimal results. *Bacillus licheniformis* and *Pseudomonas azotoformans* which were isolated from the soil of rice plants, using two potassium solubilizing bacteria, could increase the availability of potassium in the soil by 7.22 µg.mL^{-1} and 6.03 µg.mL^{-1} under optimal conditions.

In the qualitative test, the AP3.1 isolate showed the smallest clear zone. However, the quantitative test obtained the highest concentration values from day 5 to 15 (6.27, 7.47, and 8.40 µg.mL^{-1}, respectively). Moreover, a previous study isolated two bacterial strains from Tianmu mountain regions which had excellent potassium solubilizing *B. mucilagenosus* for the decomposition of silicate in liquid culture [22]. Similarly, Adeleke [23] has reported the ability of ectomycorrhizal fungi in the mobilization of P and K sources from insoluble.

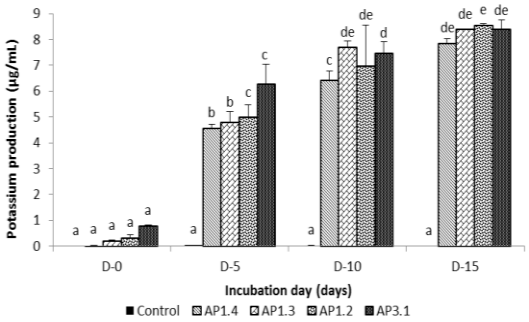


Figure 4. Quantitative test results of phosphate solubilizing bacteria in producing potassium. The different notations on this histogram indicate differences between treatments ($p < 0.05$).

The efficient KSB strains were identified as *Pseudomonas* spp., *Bacillus* spp., and *Burkholderia* spp. by following various polyphasic taxonomic approaches. KSB strains preferred acidic to a neutral range of pH, temperature, and other nutritional factors which were recorded as pH 7.0, 25°C, and 6% of NaCl concentration [24]. Several genera of potassium solubilizing bacteria (KSB) are *Azospirillum, Agrobacterium, Bacillus, Enterobacter, Erwinia, Flavobacterium, Micrococcus, Pseudomonas, Rhizobium,* and *Serratia* [20,21,25]. The mechanism of solubilizing potassium and insoluble potassium

ISSN. 2087-2852
E-ISSN. 2338-1655

minerals such as mica, illite, and ortholox is by secreting organic acids such as (citric, oxalic, tartaric, succinic, and α-ketoglucanic acids) by potassium solubilizing bacteria which directly dissolve K rock or chelate silicate ions to bring K into the dissolved form. In addition, K dissolving occurs due to the formation of complexes between organic acids secreted by bacteria and metal ions that bind to K minerals such as Fe^{2+}, Al^{3+}, and Ca^{2+} [4,6]. One of the most common signs of potassium deficiency is chlorosis of the leaf surface[24].

CONCLUSION

In this study, the AP1.3 isolate was the best as a phosphate and potassium solubilizing endophytic bacteria, which could be used as a biofertilizer. Then, the molecular identification and application of AP1.3 in plants are necessary to determine its ability to provide nutrients for plants.

REFERENCES

[1] Arifin, Z., A. Krismawati. 2008. Organic farming towards sustainable agriculture, 1st Ed. Bayumedia Publishing. Malang.

[2] Rahmah, A., M. Izzati., S. Parman. 2014. The effect of liquid organic fertilizer made from chicory (*Brassica chinensis* L.) waste on the growth of sweet corn plants (*Zea mays* L. Var. Saccharata). *Bulletin of Anatomy and Physiology*. 22(1). 65-71.

[3] Asih, P.R., M. Suharman, Giyanto. 2017. Isolation of rhizobacteria and the effect of its application with N-P fertilizer on seed quality and seedling growth of maize. *Jurnal Agronomi Indonesia*. 45(3). 255-262.

[4] Bakhshandeh, E., H. Pirdashti, K.S. Lendeh, 2017. Phosphate and potassium-solubilizing bacteria effect on the growth of rice. *Ecol. Eng*. (103). 164-169.

[5] Mursyida, E., N.R. Mubarik, A. Tjahjoleksono. 2015. Selection and identification of phosphate-potassium solubilizing bacteria from the area around the limestone mining in Cirebon Quarry. *Res. J. Microbiol*. 10(6). 270-279.

[6] Meena, V.S., B.R. Marya, J.P. Verma, S.M. Ram. 2016. Potassium solubilizing microorganisms for sustinable agriculture. Springer eBook. India.

[7] Zhang, X., Y. Zhou, Y. Li, X. Fu, Q. Wang. 2017. Screening and characterization of endophytic *Bacillus* for biocontrol of grapevine downy mildew. *Crop Prot*. 96. 173-179.

[8] Setia, I.N., Suharjono. 2015. Diversity and potential test of chitinolytic bacteria from shrimp waste. *Biotropika*. 3(2). 95-98.

[9] Lynn, T.M., H.S. Win, E.P. Kyaw, Z.K. Latt, S.S. Yu. 2013. Characterization of phosphate solubilizing and potassium decomposing strains and study on their effets on tomato cultivation. *Int. J. Innov. Appl. Stud*. 3(4). 959-966.

[10] Parmar, P., S.S. Sindhu. 2013. Potassium solubilization by rhizosphere bacteria: influence of nutritional and environmental conditions. *J. Microbiol. Res*. 3(1). 25-31.

[11] Ludwig, J.A., J.F. Reynolds. 1988. Statistical ecology a primer on methods and computing. Wiley-Interscience Pub. New York.

[12] Marag, P.S., A. Suman. 2018. Growth stage and tissue specific colonization of endophytic bacteria having plant growth promoting traits in hybrid and composite maize (*Zea mays* L.). *Microbiol. Res.* 214. 101-113.

[13] Marista, E., S. Khotimah, R. Linda. 2013. Phosphate solubilizing bacteria isolated from three types of rhizosphere soil of the Nipah banana plant (*Musa paradisiaca* var. Nipah) in the City of Singkawang. *J. Protobiont.* 2(2). 93-101.

[14] Mohanty, S.R., G. Dubey, B. Kollah. 2017. Endophytes of *Jatropha curcas* promote growth of maize. *Rhizosphere*. (3). 20-28.

[15] Sashidhar, B., A.R. Podile. 2010. Mineral phosphate solubilization by rhizosphere bacteria and scope for manipulation of the direct oxidation pathway involving glucose dehydrogenase. *J. Appl. Microbiol*. 109(1). 1-12.

[16] Hardjowigeno, S. 1992. Geology. Peatlands: potential for agriculture and environmental aspects. ICRAF. Mediyatama Sarana Perkasa. Jakarta.

[17] Pande, A., P. Pandey, S. Mehra, M. Singh, S. Kaushik. 2017. Phenotypic and genotypic characterization of phosphate solubilizing bacteria and their efficiency on the growth of maize. *J. Genet. Eng. Biotechnol.* 15. 379-391.

[18] Rajawat, M.V.S., S. Singh, S.P. Tyagi, A.K. Saxena. 2016. A modified plate assay for rapid screening of potassium solubilizing bacteria. *Pedosphere*. 26(5). 768-773.

[19] Chen, Y.P., P.D. Rekha, A.B. Arun, F.T. Shen, W.A. Lai, C.C. Young. 2006. Phosphate solubilizing bacteria from subtropical soil

and their tricalcium phosphate solubilizing abilities. *Appl. Soil. Ecol*. (34). 33-41.

[20] Walpola, B.C., M.H. Yoon. In vitro solubilization of inorganic phosphates by phosphate solubilizing microorganisms. *Afr. J. Microbiol. Res*. (27). 3534-3541.

[21] Don, N.T., C.N. Diep. 2014. Isolation, characterization and identification of phosphate and potassium solubilizing bacteria from weathered materials of Granite Rock Mountain, that Son, an Giang Province, Vietnam. *Am. J. Life Sci*. 2(5). 282-291

[22] Xiufang, H., C. Jishuang, G. Jiangfeng. 2006. Two phosphate and potassium solubilizing bacteria isolated from Tianmu Mountain, Zhejiang, China. W. *J. Microbiol. Biotechnol.* 22. 983.990.

[23] Adeleke, R.A., T.E. Cloete, A. Bertr, D.P. Khasa. 2010. Mobilization of potassium and phosphorus from iron ore by ectomycorrhizal fungi. *World J. Microbiol. Biotechnol.* 26. 190-195.

[24] Bagyalakshmi, B., P. Ponmurugan, A. Balamurugan. 2017. Potassium solubilization, plant growth promoting substances by potassium solubilizing bacteria (KSB) from southern Indian Tea plantation soil. *Biocatal. Agric. Biotechnol.* 12. 116-124.

[25] Verma, P., A.N. Yadav, K.S. Khannam, N. Panjiar, S. Kumar, A.K. Saxena, A. Suman, 2015. Assessment of genetic diversity and plant growth promoting attributes of psychrotolerant bacteria allied with wheat (Triticum aestivum) from the northern hills zone of India*. Ann. Microbiol*. 65. 1885-1899.

[26] Bashir, Z., M.Y. Zargar, M. Husain, F.A. Mohiddin, S. Kousar, A. Ahmad, J.P. Rathore. Potassium solubilizing micro-organisms: mechanism and diversity. *Int. J. Pure App. Biosci*. 5(5). 653-660.

[27] Zhang, C., F. Kong. 2014. Isolation and identification of potassium-solubilizing bacteria from tobacco rhizospheric soil and their effect on tobacco plants. *Appl. Soil Ecol.* 82. 18-25.

[28] Viruel, E., L.E. Erazzú, L. Martínez-Calsina, M.A. Ferrero, M.E. Lucca, F. Siñeriz. 2014. Inoculation of maize with phosphate solubilizing bacteria: effect on plant growth and yield. *J. Soil Sci. Plant Nutr*. 14(4). 819-831.

ISSN. 2087-2852
E-ISSN. 2338-1655

The Study of Combination Ethanol Extract of *Averrhoa bilimbi* L. and *Momordica charantia* L. on $CD4^+CD25^+TGF\text{-}\beta^+$ Spleenocytes of Hyperglycemia Mice

Harits Amrulloh[1], Bella Novinda[1], Intan Sartika[1], Sri Widyarti[2], Muhaimin Rifa'i[2]*

[1]Master Program of Biology, Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Brawijaya, Malang, Indonesia

[2]Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Brawijaya, Malang, Indonesia

Abstract

Diabetes mellitus is one of the four priority non-infectious diseases in the world. Plant-based medicine is an alternative treatment with few side effects. Star fruit (*Averrhoa bilimbi* L.) and bitter melon (*Momordica charantia* L.) are plants that have anti-hyperglycemic activity. Hyperglycemia produces Reactive Oxygen Species (ROS) that make the β-cells of the pancreas necrosis that decreasing insulin synthesis. The anti-inflammatory activity appears based on the relative levels of $CD4^+$ and $CD25^+$, which are TGF-β-producing regulatory T cells where TGF-β is a mediator that acts as an immunosuppressant. TGF-β would induce CD4 + T lymphocytes into T reg. The purpose of this study is to determine the profile of TGF-β on $CD4^+$ and $CD25^+$ spleenocytes on hyperglycemia mice after ABMC (*Averrhoa bilimbi Momordica charantia* mix) treatment. Mice were divided into 5 groups, non-diabetic (N), hyperglycemia (H), hyperglycemia with extract doses of 10 $mg.kg^{-1}$ BW (P1), 40 $mg.kg^{-1}$ BW (P2), and 160 $mg.kg^{-1}$ BW (P3). Diabetic mice were obtained after a single injection dose of 145 $mg.kg^{-1}$ BW *streptozotocin* (STZ). The result showed that ABMC can reduce blood sugar levels faster and able reduce the number of $CD4^+TGF\text{-}\beta^+$ cells in hyperglycemia mice.

Keywords: *Averrhoa bilimbi* L., $CD4^+TGF\text{-}\beta^+$, $CD25^+TGF\text{-}\beta^+$, hyperglycemia, *Momordica charantia* L.

INTRODUCTION

Hyperglycemia is characteristic of diabetes mellitus (DM) due to decreased insulin secretion by pancreatic β cells. Hyperglycemia describes an increase in blood glucose levels in circulating blood and produces Reactive Oxygen Species (ROS) through various pathways, including redox balance dysregulation, augmentation of glycation products, activation of protein kinase C which ultimately leads to oxidative stress in various tissues [1]. A survey from the World Health Organization (WHO) shows that Indonesia has a high number of DM patients, ranking fourth in the world after India, China, and America [2]. The number of people with diabetes has quadrupled from 108 million in 1980 to 422 million in 2014 [3].

The main cause of complications in diabetes mellitus is the presence of oxidative stress conditions due to increased reactive oxygen species (ROS) [4-6]. Thus the islets contain very low levels of antioxidants, so the accumulation of ROS can produce *oxidative stress*, a well-known trigger for β cell apoptosis [7]. Here, the altered β-cell redox state, coupled with other factors such as nutrient-induced augmentation of insulin synthesis, can lead to stress-induced apoptosis of the endoplasmic reticulum. The result is a reduced functional β cell mass, resulting in a further decrease in insulin secretion [8,9].

The hyperglycemia will also increase the expression of transforming growth factor-ß (TGF-ß). TGF-β signaling is one of the signaling pathways that affect β-cell differentiation and function. Impaired TGF-β signaling has the potential to be the center of β-cell dedifferentiation. TGF-ß signaling is involved in almost all tissue types in the body and has been shown to play a role in the regeneration of β-cells in the islets of the pancreas. Failure of β cells in type II diabetes is a multifaceted process that can include inflammation of the islets of Langerhans, increased β cell apoptosis, decreased β cell proliferation, and dedifferentiation of β cells to a progenitor-like state [10].

Both TGF-β and CD4+-CD25+ regulatory T cells (Treg) play important roles in controlling immune responses and maintenance of immune homeostasis. TGF-β is a pleiotropic cytokine with a number of context-dependent effects on immune cells, including inhibition of T cell proliferation and differentiation, macrophage activation, and DC maturation [11]. The role of TGF-β1 in the regulation of $CD4^+CD25^+$ Treg cells in vivo was confirmed by a study using a type I diabetes mouse model. In that study, the transient TGF-β1 pulses in islets during the early phase of diabetes were sufficient to inhibit

*Correspondence address:
Muhaimin Rifa'i
Email : Immunobiology@ub.ac.id
Address : Dept. Biology, University of Brawijaya, Veteran Malang, 65145

disease onset by stimulating expansion and expression of Foxp3 from $CD4^{+}CD25^{+}$ Treg intraislet [12].

Plant-based medicine is one of the alternatives used as diabetes therapy because of its low side effects and affordable cost. The active principles present in medicinal plants have been reported to have the ability to regenerate pancreatic β cells, release insulin and fight insulin resistance problems [13,14]. Bitter melon (*Momordica charantia* L.) has high antioxidant activity. It also has active compounds including saponins, flavonoids, polyphenols, and vitamin C, as well as insulin-mimetic compounds consisting of charantin, vicine, and polypeptide-p, which are considered the main hypoglycemic compounds [13,15,16]. Ethanol extract (95%) from *M. charantia* L. has significantly lowered blood glucose in *streptozotocin*-diabetic male albino rats at a dose of 35 $mg.kg^{-1}$ BW [17].

Star fruit *(Averrhoa bilimbi* L.) contains anti-diabetic substances, including flavonoids, saponins, and vitamin C [18]. Saponins function as anti-hyperglycemic by preventing glucose uptake at the brush border in the small intestine, while flavonoids are alpha-glucosidase inhibitors, which function to delay carbohydrate absorption. The ethanol extract of the fruit and leaves of starfruit can reduce blood glucose in diabetic rats [19]. The ethanol extract of leaves and fruit from *A. bilimbi* L showed a very significant anticoagulant effect in normal and diabetic male Wistar rats by giving it for 14 consecutive days [20].

M. charantia L. has high antioxidant activity but takes longer to lower blood glucose levels, while *A. bilimbi L*. can lower blood glucose levels quickly but has low antioxidant activity. Based on this description, scientific research is needed to be related to the use of a combination of *M. charantia* L. and *A. bilimbi* L. extracts on blood sugar levels, transforming growth factor-ß (TGF-β) of the spleen in mice (*Mus musculus*) hyperglycemia model.

MATERIAL AND METHOD

Preparing Combination Extract ABMC *(Averrhoa bilimbi-Momordica charantia)*

A. bilimbi L. and *M. charantia* L. fruit were processed to become *simplicia* at Balai Materia Medica, Batu, East Java. The *simplicia* extraction of *M. charantia* L. and *A. bilimbi* L. fruit used 96% ethanol [20]. Soaking was carried out with 1 liter of ethanol and stirred, followed by filtering after maceration for 1 x 24 hours, and replaced with new ethanol solvent. This step was carried out 3 times. The macerated solution obtained is evaporated in a rotary evaporator for 1-2 hours until all the solvent evaporates. The extract was then further dried using freeze-dry to evaporate the remaining solvent. The dried extract is stored in a refrigerator at 4°C.

Animals

The research got approval from the ethics commission No. 1109-KEP-UB 2020. This study used 8-10 weeks old female Balb/c mice (*Mus musculus*) with an average weight of 25 grams obtained from the Animal Physiology Laboratory of the State Islamic University of Maulana Malik Ibrahim Malang.

Hyperglycemia Mice

Streptozotocin (STZ) was injected intraperitoneally at a single dose of 145 $mg.kg^{-1}$ BW [22]. The induction of STZ-induced diabetes was confirmed by measuring the blood glucose levels. Mice with glucose levels above 200 $mg.dL^{-1}$ were subjected to further treatment using ABMC.

Abmc Treatment

The diabetic mice were randomly divided into five groups consisting of five animals each. Group N is the non-diabetic (control), Group H is the diabetic mice. Group P1, P2, and P3 are diabetic mice who got ABMC 10, 40, and 160 $mg.kg^{-1}$ BW respectively for 14 days orally. Blood glucose levels were measured on days 1 and 15 after [16,20].

Spleenocytes Isolation

The mice were killed by cervical dislocation then placed on the operating table with a dorsal position and all four limbs fixed. Then an incision was made on the left side of the mice's abdomen. The spleen organs were taken and rinsed using PBS twice, homogenized by crushing, put in a different petri dish. Homogenate was centrifuged at 2500 rpm for 5 minutes 4°C. The pellets were resuspended in 1 mL of PBS.

Antibody Staining

A total of 100 µL of cell suspension was added with 500 µL of PBS, centrifuged at 2500 rpm for 5 minutes at 4°C. The pellets were added 50 µL of anti-CD4 and anti-CD25, incubated for 30 minutes at 4°C. Then, the suspension was added Cytoperm/Cytofix kit according to the manufacturer's protocol (BDBiosciences Pharmingen) and modified by Rifa'i and Widodo [23]. After centrifugation, the pellet was

ISSN. 2087-2852
E-ISSN. 2338-1655

incubated with FITC-conjugated rat-antimouse CD4, PE-conjugated rat-antimouse CD25, and PE/Cy5 conjugated rat-antimouse TGF-β (from BDBiosciences Pharmingen), respectively at 4°C for 20 minutes. The cell was resuspended with 400 µL PBS and running to *flow cytometry* (BDCellquest ProTM Software).

Data Analysis

Statistical Data was analyzed using one-way ANOVA (Analysis of Variance) with α 5% followed by Tukey Test Honestly Significant Difference (HSD) test to evaluate the significant difference among treatments.

RESULT

ABMC Decrease The Blood Glucose Level on Diabetic Mice

The non-diabetic mice had blood sugar levels of 143±7.24 mg.dL^{-1} ($p < 0.05$) on the first day and 145±4.20 mg.dL^{-1} on day 15, whereas the hyperglycemia mice had blood sugar levels of 462±15.17 mg.dL^{-1} on the first day and 412±9.78 mg.dL^{-1} on day 15. There was a decrease blood glucose level in group P1 from 439±11.64 mg.dL^{-1} on the first day and became 346±6.18 mg.dL^{-1} on day 15 (21.1 %), P2 was 436±9.35 mg.dL^{-1} on the first day and 295±6.16 mg.dL^{-1} on day 15 (32.3 %) and P3 430±11.05 mg.dL^{-1} on the first day and 345±15.54 mg.dL^{-1} on day 15 (19.7 %) (Fig. 1). On day 1, hyperglycemia control was not significant with the treatment group because the ABMC treatment needed time to lower blood glucose. On day 15, the treatment groups were significantly decreased blood glucose level faster-compared hyperglycemia control, as shown by the stars above the graph in Figure 1.

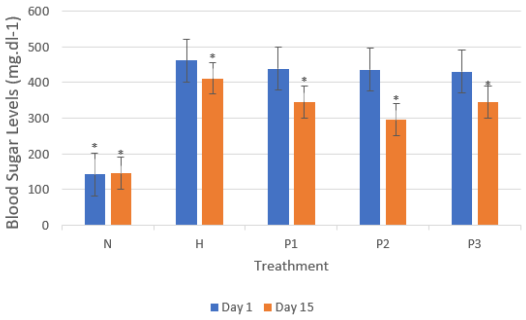


Figure 1. The decreasing fasting blood sugar levels.
Description:
N = non-diabetic
H = hyperglycemia control
P1 = hyperglycemia mice + treatment 10 mg.kg $^{-1}$ BW
P2 = hyperglycemia mice + treatment 40 mg.kg^{-1} BW
P3 = hyperglycemia mice + treatment 160 mg.kg^{-1} BW

ABMC Cannot Decrease The Number CD25^{+}TGF-β^{+} Cells

The hyperglycemia mice (H) had an average number of CD25^{+}TGF-β^{+} 0.47%±0.09 ($p < 0.05$), not significantly different from non-diabetics (N) and all treatment groups as shown with no stars above the graph (Fig. 2). Meanwhile, group P1 had an average relative number of CD25+TGF-β+ 0.55%±0.08, which was significantly different from group P3 with an average relative number of CD25^{+}TGF-β^{+} cells 0.38%±0.05. So statistically ABMC cannot decrease the number of CD25^{+}TGF-β^{+} cells.

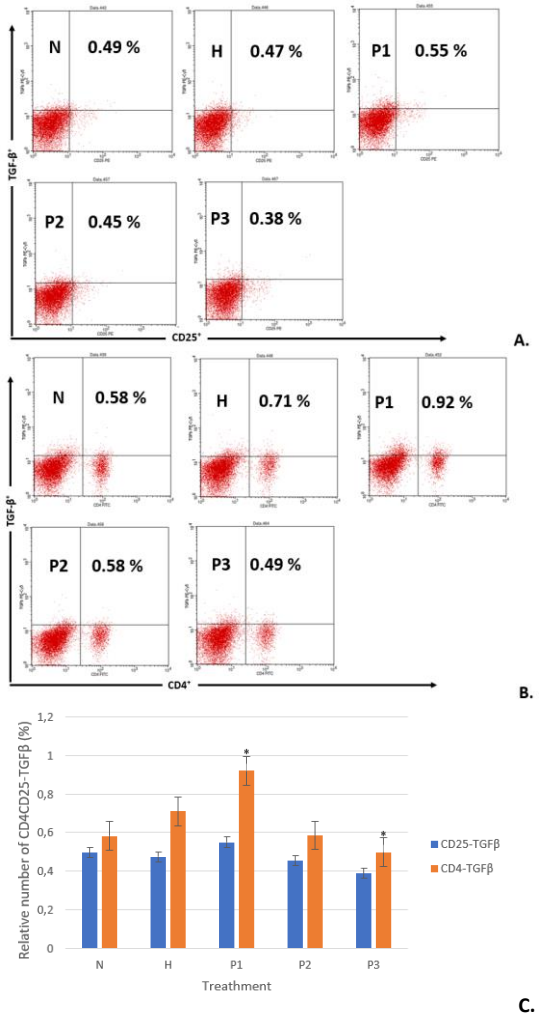


Figure 2. The number of CD4^{+}TGF-β^{+} and CD25^{+}TGF-β^{+} cells in hyperglycemia mice.
A. Dot Plot of CD4^{+}TGF-β^{+} cells number
B. Dot Plot of CD25^{+}TGF-β^{+} cells number
C. Relative Number of CD4^{+}TGF-β^{+} and CD25^{+}TGF-β^{+}
Description:
N = non-diabetic
H = hyperglycemia control
P1 = hyperglycemia mice + treatment 10 mg.kg $^{-1}$ BW
P2 = hyperglycemia mice + treatment 40 mg.kg^{-1} BW
P3 = hyperglycemia mice + treatment 160 mg.kg^{-1} BW

ISSN. 2087-2852
E-ISSN. 2338-1655

ABMC Decrease The Number $CD4^+TGF\text{-}\beta^+$ Cells

The hyperglycemia control (H) had an average relative number of $CD4^+TGF\text{-}\beta^+$ cells 0.71%±0.06 ($p < 0.05$) but not significantly different from the non-diabetic (N) and treatment group 2 (P2), which has an average relative number of $CD4^+TGF\text{-}\beta^+$ 0.58%±0.08. Treatment group 1 (P1) had the highest average number of $CD4^+TGF\text{-}\beta^+$ cells 0.92%±0.05. Treatment group 3 (P3) had the lowest average number of $CD4^+TGF\text{-}\beta^+$ 0.49%±0.07 and significantly different from the hyperglycemia control (H), as shown by the stars above the graph (Fig. 2).

DISCUSSION

Streptozotocin (STZ) causes toxicity to pancreatic-β cells by damaging DNA through the mechanism of Poly-ADP ribosylation polymerase (PARP) activation, decreasing cellular NAD+ and ATP. Diabetogenic effects of streptozotocin are also initiated by reactive oxygen species (ROS) via a direct toxic effect on GLUT 2 [20]. High levels of ROS will inhibit T cell proliferation, which leads to apoptosis and interferes with the process of T cell differentiation and regulation of its function. Disruption in the process of differentiation and proliferation of T cells will cause a decrease in the number of regulatory T cells, which are a subset of cluster differentiated T cells (CD) 4^+ that maintain peripheral tolerance and suppress adaptive immune responses by secreting anti-inflammatory cytokines, such as Transforming growth factor-ß (TGF-β). $CD4^+CD25^+$ Treg cells can inhibit the inflammatory response through various pathways, such as increasing the secretion of anti-inflammatory cytokines, modulating the microenvironment, and altering cell receptor expression [20,21,24].

TGF-β signaling is one of the signaling pathways that affect β-cell differentiation and function. The disruption of TGF-β signaling has the potential center of β-cell dedifferentiation. Intact TGF-β signaling can modulate β-cell response to increase glucose levels. TGF- β can also maintain Foxp3 expression in $CD4^+CD25^+$ Treg to enhance immunosuppressive function [25].

In Figure 2, Treatment ABMC extract can decrease the number of $CD4^+TGF\text{-}\beta^+$ cells on hyperglycemia mice. TGF-β is a multifunctional cytokine that plays various roles in cellular differentiation and immune regulation [25]. TGF-β regulates the inflammatory response through activation control or chemotaxis, and the survival of various immune cells such as lymphocytes, natural killer cells, dendritic cells, macrophages, mast cells, and granulocytes [26,27].

The *momordicine* 1 found in *M. charantia* L. fruit can significantly improve glucose-induced ROS by activating the Nrf2/HO-1 pathway. Momordicine 1 can inhibit fibrogenesis through modulation of Nrf2-mediated TGF-β1-Smad2/3 signal transduction [28]. While the *charantin* compound from *M. charantia L.* fruit can stimulate the pancreas-β cells to produce insulin, increase glycogen sugar reserve deposits in the liver. In addition, there are polypeptide-P insulin compounds that directly reduce blood glucose levels [29].

Starfruit contains flavonoids, triterpenoids, and quercetin. Flavonoids are active antihipoglycemic compounds by stimulating insulin secretion [28,29]. Also, the flavonoid is alpha-glucosidase inhibitors which function to delay carbohydrate absorption [30]. Meanwhile, quercetin can improve kidney function in diabetic nephropathy rats by inhibiting the overexpression of TGF-β1 and CTGF [31].

CONCLUSION

ABMC combination able reduce 19.7%-32.3% blood glucose levels on hyperglycemic mice. ABMC dose of 40 and 160 $mg.kg^{-1}$ BW can decrease (18.3-30.9%) the number of $CD4^+TGF\text{-}\beta^+$ cells.

REFERENCES

[1] Pitocco, D., M. Tesauro, R. Alessandro, G. Ghirlanda, C. Cardillo. 2013. Oxidative stress in diabetes: implications for vascular and other complications. *Int. J. Mol. Sci.* 14(11). 21525–21550.

[2] World Health Organization. 2016. Global Report on Diabetes.

[3] Department of Health, Republic of Indonesia. 2014. Pedoman pengendalian diabetes melitus dan penyakit metabolik. Department of Health, Republic of Indonesia. Jakarta.

[4] Ullah, A., A. Khan, I. Khan. 2016. Diabetes mellitus and oxidative stress-A concise review. *Saudi Pharm. J.* 24(5). 547–553.

[5] Johansen, J.S. 2005. Oxidative stress and the use of antioxidants in diabetes: Linking basic science to clinical practice. *Cardiovasc. Diabetol.* 4. 5.

[6] Ferrari, C.K.B., E.A.E.S. Torres. 2003. Biochemical pharmacology of functional foods and prevention of chronic diseases of aging. *Biomed. Pharm.* 57. 251-260.

ISSN. 2087-2852
E-ISSN. 2338-1655

[7] Poitout, V., R.P. Robertson. 2008. Glucotoxicity: fuelexcess and beta cell dysfunction. *Endocr. Rev*. 29(3). 351-366.
[8] Eizirik, G.l., A.K. Cardozo, M. Cnop. 2008. The role for endoplasmic reticulum stress in diabetes millitus. *Endocr. Rev.* 29. 42-61.
[9] Dor, Y., B. Glaser. 2013. β-cell dedifferentiation and type 2 diabetes. *N. Engl. J. Med.* 368. 572-573.
[10] Fischbach, S., G.K. Gitters. 2014. The role of tgf-β signaling in β-cell dysfunction and type 2 diabetes: A review. *J. Cytol. Histol*. 5.6.
[11] Gorelik, L., R.A. Flavell. 2002. Transforming growth factor beta in T-cell biology. *Nat. Rev. Immunol.* 2. 46-53.
[12] Peng, Y., Y. Laouar, M.O. Li, E.A. Green, R.A. Flavell. 2004. TGF-beta regulates in vivo expansion of Foxp3- expressing CD4+CD25+ regulatory T cells responsible for protection against diabetes. *Proc. Natl. Acad. Sci. USA*. 101. 4572-4577.
[13] Joseph, B., D. Jini. 2013. Antidiabetic effects of *Momordica charantia* (bitter melon) and its medicinal potency'. *Asian Pac. J. Trop. Dis.* 3(2). 93–102.
[14] Hui, H., G. Tang, V.L.W. Go. 2009. Hypoglycemic herbs and their action mechanisms. *Chin. Med.* 4. 11-14.
[15] Nagy, M.A., M.A. Bastawy, N.M. Abdel-Hamid. 2012. Effects of *Momordica charantia* on streptozotocin-induced diabetes in rats: role of insulin, oxidative stress and nitric oxide. *J. Health Sci.* 2(2). 8–13.
[16] Mahmoud, Mona F., F.E. Zahraa, El. Ashry, Maraghy, N. El. Nabila, A. Fahmy. 2017. Studies on the antidiabetic activities of *Momordica charantia* fruit juice in streptozotocin-induced diabetic rats. *Pharm. Biol*. 55(1). 758-765.
[17] Gupta, R., S. Kant, S. Johri, A.M. Saxena. 2007. Effect of ethanolic extract of *Momordica charantia* on blood sugar level in normal and *streptozotocin* induced diabetic rats. *J. Appl. Biosci.* 33(2). 153-155.
[18] Kurup, S.B., S. Mini. 2017. Protective potential of *Averrhoa bilimbi* fruits in ameliorating the hepatic key enzymes in streptozotocin-induced diabetic rats. *Biomed. Pharmacother.* 85. 725-732.
[19] Pushparaj, P.N., K.H.T. Benny, H.T. Chee. 2001. The mechanism of hypoglycemic of the semi-purified fractions of *Averrhoa bilimbi* in streptozotocin-diabetic rats. *Life Sci.* 70. 535-547.
[20] Nurafifah, D., H. Hashim, N. Samsulrizal. 2013. Anticoagulant activity of *Averrhoa bilimbi* Linn in normal and alloxaninduced diabetic rats. *The Open Conference Proceedings Journal*. 4. (Suppl-2, M6) 21-26. DOI: 10.2174/2210289201304020021.
[21] Qiao, Y C., J. Shen, L. He, X. Hong, F. Tian, Y.H. Pan. 2016. Changes of regulatory T cells and of proinflammatory and immunosuppressive cytokines in patients with type 2 diabetes mellitus: A systematic review and meta-analysis. *J. Diabetes Res*. 1-19. DOI: 10.1155/2016/3694957.
[22] Akbarzadeh, A., D. Norouzian, M.R. Mehrabi, Sh. Jamshidi, A. Farhangi, A.A. Verdi, S.M.A. Mofidian, B.L. Rad. 2007. Induction of diabetes by streptozotocin in rats. *Indian J. Clin. Biochem*. 22(2). 60–64.
[23] Rifa'i, M., N. Widodo. 2014. Significance of propolis administration for homeostasis of CD4+CD25+ T immunoregulatory cells controlling hyperglycemia. *SpringerPlus*. 3. 526.
[24] Erwin, E., E. Etriwati, M. Muttaqien, T.W. Pangestiningsih, S. Widyarini. 2013. Ekpressi insulin pada pankreas mencit (*Mus musculus*) yang diinduksi dengan streptozotocin berulang. *Jurnal Kedokteran Hewan.* 7(1). 97-100.
[25] Sarma, A.D., A.R. Mallick, A. Ghosh. 2010. Free radicals and their role in different clinical conditions : an overview. *Int. J. Pharm. Sci. Res.* 1(3). 185–192.
[26] Li, M.O., Y.Y. Wan, S. Sanjabi, A.K.L. Robertson, R.A. Flavell. 2006. Transforming Growth Factor-β regulation of immune responses. *Annu. Rev. Immunol.* 24(1). 99-146.
[27] Sanjabi, S., S.A.Oh, M.O. Li. 2017. Regulation of the immune response by TGF-β: from conception to autoimmunity and infection. *Cold Spring Harb. Perspect. Biol.* 9. a022236. DOI: 10.1101/cshperspect.a022236
[28] Chen, P.Y., N.L. Shih, W.R. Hao, C.C. Chen, J.C. Liu, L.C. Sung. 2018. Inhibitory effects of Momordicine I on high-glucose-induced cell proliferation and collagen synthesis in rat cardiac fibroblasts. *Oxid. Med. Cell. Longev.* Article ID 3939714.
[29] Tsabang, N., L.W.T Djeufack, C.G. Yedjou, P.B. Tchounwou. 2019. Importance of food plants in the prevention and treatment of diabetes in Cameroon. *Bioact. Compd. Health Dis*. 2(2). 11–26. DOI: 10.31989/ bchd.v2i2.554.

[30] Nwachukwu, D.C. 2010. Investigation of the anti-hyperglycaemic effect of the leaf extracts of *Solanum dulcamara* in diabetic rats. *Indian J. Nov. Drug Deliv*. 2(4). 138-142.
[31] Lai, P.B., L. Zhang, L.Y. Yang. 2012. Quercetin ameliorates diabetic nephropathy by reducing the expressions of transforming growth factor-β1 and connective tissue growth factor in streptozotocin-induced diabetic rats. *Ren. Fail.* 34(1). 83-87.

ISSN. 2087-2852
E-ISSN. 2338-1655

MANUSCRIPT SUBMISSION

FOCUS AND SCOPE

Journal of Experimental Life Science (JELS) is scientific journal published by Graduate Program of Brawijaya University as distribution media of Indonesian researcher's results in life science to wider community. JELS is published in every four months. JELS published scientific papers in review, short report, and life sciences especially nanobiology, molecular biology and cellular biology. JELS is scientific journal that published compatible qualified articles to academic standard, scientific and all articles reviewed by expert in their field.

Journal of Experimental Life Science (JELS) have vision to become qualified reference media to publish the best and original research results, and become the foundation of science development through invention and innovation on cellular, molecular, and nanobiology rapidly to community.

Journal of Experimental Life Science (JELS) have objectives to published qualified articles on research's results of Indonesian researchers in life science scope. JELS encompasses articles which discuss basic principles on nature phenomenon with cellular, molecular, and nanobiology approach.

PEER REVIEW PROCESS

Publication of articles by JITODE is dependent primarily on their validity and coherence, as judged by peer reviewers, who are also asked whether the writing is comprehensible and how interesting they consider the article to be. All submitted manuscripts are read by the editorial staff and only those articles that seem most likely to meet our editorial criteria are sent for formal review. All forms of published correction may also be peer-reviewed at the discretion of the editors. Reviewer selection is critical to the publication process, and we base our choice on many factors, including expertise, reputation, and specific recommendations. The editors then make a decision based on the reviewers' advice, from among several possibilities:

Accepted, with or without editorial revisions
Invite the authors to revise their manuscript to address specific concerns before a final decision

Rejected, but indicate to the authors that further work might justify a resubmission

Rejected outright, typically on grounds of specialist interest, lack of novelty, insufficient conceptual advance or major technical and/or interpretational problems

PUBLICATION FREQUENCY

JELS publish 2 Issues per year until 2017. JELS started to publish 3 Issues per year since 2018.

OPEN ACCESS POLICY

This journal provides immediate open access to its content on the principle that making research freely available to the public supports a greater global exchange of knowledge.

COPYRIGHT NOTICE

Authors who publish with this journal agree to the following terms:

Authors retain copyright and grant the journal right of first publication with the work simultaneously licensed under a Creative Commons Attribution License that allows others to share the work with an acknowledgement of the work's authorship and initial publication in this journal.

Authors are able to enter into separate, additional contractual arrangements for the non-exclusive distribution of the journal's published version of the work (e.g., post it to an institutional repository or publish it in a book), with an acknowledgement of its initial publication in this journal.

Authors are permitted and encouraged to post their work online (e.g., in institutional repositories or on their website) prior to and during the submission process, as it can lead to productive exchanges, as well as earlier and greater citation of published work (The Effect of Open Access).

PRIVACY STATEMENT

The names and email addresses entered in this journal site will be used exclusively for the stated purposes of this journal and will not be made available for any other purpose or to any other party.

ETHICS PUBLICATION

Research that using animal, human, and clinical testing is should already have ethical clearance certificate from authorized institution.

Title Typed in Bold, Capitalize each First Letter of Each Word, Except Conjunctive, *Scientific name* should not be Abbreviated (Calibri 14 Bold Center, should not exceed 12 words, except conjuctive)

First Author[1]*, Second Author[2], Third Author[3] (Calibri 12 Center, without title)

[1]First Author Affiliation, Correspondence author should be indicated by * symbol (Calibri 9 Center)
[2]Department of Biology, Faculty of Mathematics and Natural Sciences, University of Brawijaya, Malang, Indonesia
[3]Laboratorium of Physiology, Faculty of Medicine, University of Brawijaya, Malang, Indonesia

Abstract (Calibri 9 Bold Center)

This article illustrates preparation of your paper using MS-WORD (.doc or .rtf). Manuscript was numbered consecutively. Main text typed in two columns (67 characters), except title and abstract in one column. The manuscript should be written in English. The length of manuscript should not exceed 10 pages including table and figure in this format using A4 paper single space. The text should be in the margin of 3 cm up, down and left side, 2.5 cm on right side. Abstract includes the research purposes, research method and research results in one paragraph of *essay,* not *enumerative*. No citation in abstract. Abstract should not exceed 200 words. Keywords typed after abstract. (Calibri 9 Justify).

Keywords: manuscript, English, format, 5 words maximum (Calibri 9 Left)

INTRODUCTION* (Calibri 10 Bold, Left, Capslock)

All submitted manuscripts should contain original research which not previously published and not under consideration for publication elsewhere. Articles must be written in ENGLISH and manuscripts may be submitted for consideration as research report articles, short reports or reviews.

The introduction explains the background of the problem, the study of literature and research purposes. Some initial introduction paragraphs explain the problem and background to these problems [1]. The next few paragraphs explain the study of literature that contains recent knowledge development which is directly related to the issues. The last paragraph of the introductory section contains a description of the purposes of the study. (Calibri 10 Justify)

MATERIAL AND METHOD (Calibri 10 Bold, Left, Capslock)

This section describes the types of methods (qualitative, quantitative or mixed-method) with details of methods of data collection and data analysis [2]. This section also describes the perspective that underlying the selection of a particular method. (Calibri 10 Justify)

Correspondence address: (Calibri 8 Bold, Left)
Full name of correspondence author
Email : sapto@jurnal.ub.ac.id
Address : afiliation address include post code

Data Collection (Calibri 10 Bold, Left)

Explain the data collection methods, i.e. surveys, observations or archive, accompanied by details of the use of such methods. This section also describes the population, sampling and sample selection methods. (Calibri 10 Justify)

The use of English language should followed proper grammar and terms. Name of organism shoul be followed by its full scientific name in the first mention, in *italic* [3]. Author of the scientific name and the word of "var." typed regular. Example: *Stellaria saxatillis* Buch. Ham. First abbreviation typed in colon after the abbreviated phrase.

Author must use International Standard Unit (SI). Negative exponent used to show the denominator unit. Example: g l^{-1}, instead of g/l. The unit spaced after the numbers, except percentage [4]. Example: 25 g l^{-1}, instead of 25gl^{-1}; 35% instead of 35 %. Decimal typed in dot (not coma). All tables and figures should be mentioned in the text.

RESULT AND DISCUSSION (Calibri 10 Bold, Left, Capslock)

This section contains the results of the analysis and interpretation or discussion of the results of the analysis. Describe a structured, detailed, complete and concise explanation, so that the reader can follow the flow of analysis and thinking of researchers [5]. Part of the results study should be integrated with the results of the

analysis and the results and discussion are not separated.

Table

Table should be submitted within the manuscript and in separated file of *Microsoft Excel* (xls.). Table whould not exceed 8 cm (one column) and 17 cm (two columns). Table should be embedded in different page after references.

Table should be numbered in sequence. Table title should be brief and clear above the table, with uppercase in initial sentence. Vertical line should not be used. Footnote use number with colon and superscripted. Symbol of (*) or (**) was used to show difference in confidence interval of 95 and 99%.

Table 1. Example of the Table (Calibri 8.5 Left)

No	Point (Calibri 8.5 Justify)	Description
1		
2		
3		
4		
5		

Sources: Journal of PPSUB (Calibri 8.5 Left)

Figures

Figures should be in high resolution and well contrast in JPEG or PDF with the following conditions:

- Monochrome image (line art), figures of black and white diagram (solid/no shades of gray), resolution 1000-1200 dpi (dot per inch).
- Combination Halftone, combine figure and text (image containing text) and coloured graphic or in grayscale format. Resolution 600-900 dpi.
- Halftone, coloured figure or grayscale format without text. Resolution 300 dpi.
- Black and white figure should be in the grayscale mode, while coloured figures should be in RGB mode.
- Figure should not exceed the width of 8 cm (one column), 12.5 cm (1.5 columns) or 17 cm (two columns).
- Figures title typed clearly below the figure.
- Figure with pointing arrow should be grouped (grouping).
- Figures were recommended in black and white.
- Legend or figure description should be clear and complete. If compressed, the figure should be readable.
- Statistic graphic should be supplemented with data sources.
- If the figures come from the third party, it should have the copyright transfer from the sources.

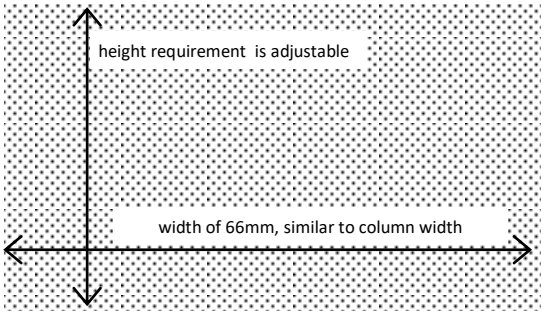


Figure 1. Illustration of Dimensional Figure of one column width. Figure dimension adjusted to the width of one column. Name the figure (diagram) written below the image. (Calibri 8.5 Justify)

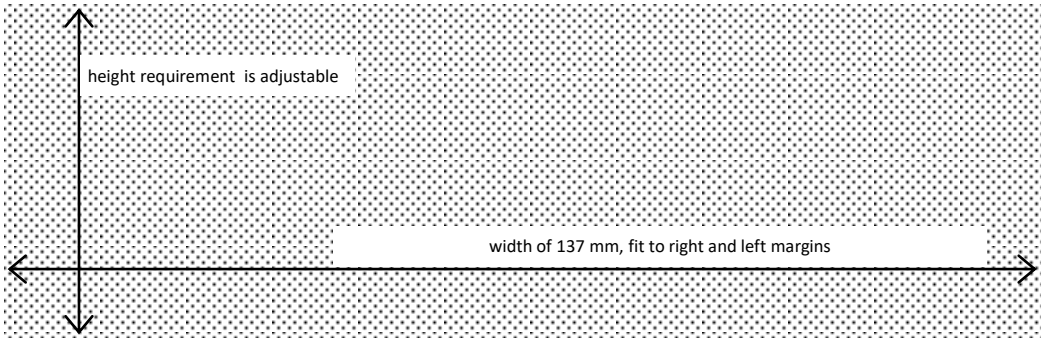


Figure 2. . Illustration of Dimensional Figure of two column width. Figure dimension adjusted to the width of two columns (137 mm). Figure were align top or bottom of the page. (Calibri 8.5 Justify)

References

1. Primary references include journal, patent, dissertation, thesis, paper in proceeding and text book.
2. Avoid self citation.
3. Author should avoid reference in reference, popular book, and internet reference except journal and private ana state institution.
4. Author was not allowed to use abstract as references.
5. References should been published (book, research journal or proceeding). Unpublished references or not displayed data can not be used as references.
6. References typed in numbering list (format number 1,2,3,…), ordered sequentially as they appear in the text (system of Vancouver or author-number style).
7. Citation in the manuscript typed only the references number (not the author and year), example: Obesity is an accumulation of fat in large quantities which would cause excessive body weight (overweight) [1]. Obesity is a risk factor of diabetic, hypertension dan atherosclerosis [2].

CONCLUSION (Calibri 10 Bold, Left, Capslock)

Conclusion of the study's findings are written in brief, concise and solid, without more additional new interpretation. This section can also be written on research novelty, advantages and disadvantages of the research, as well as recommendations for future research.(Calibri 10 Justify)

ACKNOWLEDGEMENT (Calibri 10 Bold, Left, Capslock)

This section describes gratitude to those who have helped in substance as well as financially. (Calibri 10 Justify)

REFERENCES (Calibri 10 Bold, Left, Capslock)

[1]. (Calibri 10 Justify, citation labelling by references numbering)
[2]. Vander, A., J. Sherman., D. Luciano. 2001. Human Physiology: The Mecanisms of Body Function. McGraw-Hill Higher Education. New York. (Book)
[3]. Shi, Z., M. Rifa'i, Y. Lee, K. Isobe, H. Suzuki. 2007. Importance of CD80/CD86-CD28 interaction in the recognation of target cells by $CD8^{+}CD122^{+}$ regulatory T cells. *Journal Immunology.* 124. 1:121-128. (Article in Journal)
[4]. Syafi'i, M., Hakim, L., dan Yanuwiyadi, B. 2010. Potential Analysis of Indigenous Knowledge (IK) in Ngadas Village as Tourism Attraction. pp. 217-234. In: Widodo, Y. Noviantari (eds.) Proceed-ing *Basic Science National Seminar 7* Vol.4. Universitas Brawijaya, Malang. (Article within conference proceeding)
[5]. Dean, R.G. 1990. Freak waves: A possible explanation. p. 1-65. *In* Torum, A., O.T. Gudmestad (eds). Water wave kinetics. CRC Press. New York. (Chapter in a Book)
[6]. Astuti, A.M. 2008. The Effect of Water Fraction of *Stellaria* sp. on the Content of TNF-α in Mice (*Mus musculus* BALB-C). Thesis. Department of Biology. University of Brawijaya. Malang. (Thesis)

Cover Image
3D Structure of EGCG (Epigallocatechin-3-Gallate)
Green Tea Component
Created by ::
Prof. Widodo, S.Si.,M.Si.,Ph.D MED Sc.

Address :
Building B, 1st Floor, Graduate Program Universitas Brawijaya
Jl. Mayor Jenderal Haryono 169, Malang, 65145,
East Java, Indonesia
Tel.: (+62341) 571260; Fax: (+62341) 580801
Email: jels@ub.ac.id
Web: jels.ub.ac.id

9 772087 285007