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Wheat (*Triticum aestivum* L.) mutants throught in vitro selection tolerant on lowland tropic[1]

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Key words: *: Induced mutation, wheat (Triticum aestivum), EMS, in vitro selection, regeneration.*

Abstract

Wheat engineering to improve genetic character and to increase genetic variability for lowland tolerant was not yet give superior inbred lines. The improvement of genetic variability depends on introduction lines from Turkey, India and Mexico that is highland tolerant. Wheat plants derived from the subtropics. This plant will be develops in Indonesia. The goal of this research is to engineered lowland wheat. The research was done at BB-Biogen plant tissue culture laboratory from July 2011 until December 2013. Six varieties were used such as Dewata, Selayar, Alibey, Oasis, Rabe and HP1744. This research consists of 4 stages. The first stage was the production of best callus on MS medium containing 3 gr/l 2.4-D (the best two varieties was choose). The second stage was induced mutation of embryogenic callus using EMS. The third stage was in vitro selection at temperature 27–35°C. The last stage was callus regeneration from in vitro selection. The best result for callus production was 76% for Dewata variety and 70% for Selayar variety. The higher concentration of EMS and the longer the soaking time used decreased callus growth percentage. LC_{50} of Dewata variety was 0.3% EMS at 30 minutes and LC_{50} of Selayar variety was 0.1% EMS at 60 minutes. Dewata and Selayar variety have tolerant at temperature 27°C with value of 4.2 and 3.6. The higher the temperature the more diminished the tolerant adaptation of the plants. At higher temperature, callus growth inhibited. Even at the highest temperature (35°C) callus did not grow and die. Highest number of regenerated shoots produced on media RG2 (medium MS containing 0.1 mg/l BA, 2 mg/l kinetin, 0.05 g/l tyrosine, 6% sorbitol and 3% sugar) at 1.8 and 2.2.

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Introduction

Improvement in wheat (*Triticum aestivum* L) plants is required due to limited genetic resources of tropical wheat. In vitro strategy, mutation and selection can help genetic variation obtain genetic diversity. The combination of in vitro mutation breeding and culture as well as in vitro selection increased the variation of genetic diversity that produced a superior variety and help a genetic diversification program (Jain 2010). The breeding combination has been proven to make induction and selection of mutation more effective and efficient (Maluszynski *et al.* 1995). In vitro culture technique is essential in inducing mutants, somaclonal diversity that occurs through callus culture, making it very appropriate for a breeding program. In vitro culture in the form of callus is also meristematic, making it more responsive to radioactive compared with mature cells. Callus formation is generally in plants, depending on the genotype, network type, ZPT and the media used (Bahieldin *et al.*, 2000, Rashid *et al.* 2002). According to Sarker and Biswas (2002), another criterion for the formation of callus is the selection of explants which are productive like mature embryo, immature embryo, seed, endosperm, shoots, leaves and root tips, which also affect the wheat plant regeneration in vitro.

In vitro mutation induction on vegetatively propagated plants is very effective in reducing the formation of Kimera and accelerating, desired vitro selection, and increasing the diversity of plants in a short time without changing the characteristics of the parent (Maluszynski *et al.* 1995). The use of callus derived from embryos (single cells) will help obtain a solid mutant which can avoid the formation of kimera. One of the most potential mutagens is a chemical mutagen, that is, Ethyl Methane Sulfonate (EMS) (Medina *et al.* 2005). It is the most effective and widely used in various types of organisms, ranging from viruses to mammals. EMS is often used in research because it is easy to obtain, inexpensive and non-mutagenic after being hydrolyzed (Van Harten 1998). The use of EMS to increase the occurrence of mutation has been reported, such as to produce wheat plants that fast flower and the grains that get mature quickly (Vismanathan & Reddy 1996), and to obtain M3 wheat plants that have high productivity compared to controls (Sakin *et al.* 2002). Mutation induction followed by the selection of resistance to high temperature in vitro was carried out in potato and garlic and it successfully obtained high temperature tolerant mutants (Das *et al.* 2000). The plants as a result of tissue regeneration that can overcome in vitro selection conditions are expected to have a phenotype tolerant to high temperatures and can eventually adapt to the lowlands in Indonesia.

This research needs to be done because so far there has been no previous research on mutation induction of embryonic callus using EMS and in vitro selection. The use of EMS is expected to induce resistance to high temperatures, so that the technique can support efforts to get a new mutant variety that is adaptive to tropical lowlands.

Materials and methods

The study was conducted in the Laboratory of Biological Cell and Tissue in Research and Development Center for Biotechnology and Genetic Resources (BB-Biogen) from July 2011 to December 2013. The plants used in this study were immature embryos isolated from the seeds of 6 wheat genotypes, namely Selayar, HP 1744, Alibey, Dewata, Oasis and Rabe. The media used was the basic media of MS + ZPT. The study was conducted in four stages: 1) Induction of forming the best embryogenic callus using the method of Purnamaningsih 2010. 2) Mutation induction in embryogenic callus using EMS. 3) In vitro selection at temperatures of 27°C - 35°C. 4) Callus regeneration of putative mutants as a result of in vitro selection.

The Best Embryogenic Callus Induction

The study was conducted to produce the best embryogenic callus which will be used as the material for the induction of mutation. The plant material used was immature embryos isolated from wheat seeds aged ± 3 weeks after anthesis. Before being sterilized, the wheat seeds were first peeled until clean and

washed with sterile distilled water. The sterilized seeds were placed on filter paper before the embryo was isolated by removing it from the seed. ± 1-2 mm sized embryos were planted in callus induction media with skutelum position facing up. The callus induction media was Murashige and Skoog (MS) + 2.4D 3 mg/l, sugar 3%, phytagel 3 gr/l and a pH media of 5.8 (Purnamaningsih 2010). The media in which the embryo had been planted was then put on the culture shelf in the culture room in the dark condition. The study was conducted with a completely randomized factorial design with a single factor, that is, genotypes with 6 levels: Selayar, HP 1744, Alibey, Dewata, Oasis and Rabe. The treatment consisted of 10 replicates, and each replicate comprised 10 bottles with embryonic explants. The data were analyzed using SAS 9.1 program and if there was a significant difference, the analysis would be continued using DMRT (Duncan's Multiple Range Test) at the level of 5%. Observations were made every week until the calluses reached the age of 2 weeks, covering the best percentage of embryogenic callus growth and callus diameter. Of the six genotypes to be tested, 2 genotypes were selected to produce the best embryogenic callus. The selected genotypes which would be used as the test were selected materials for further research.

Mutation Induction in Embryogenic callus using EMS

This study was carried out to seek for Lethal Concentration 50 (LC_{50}) at some EMS concentrations and soaking time periods of explants. The explants used were embryogenic calluses of 2 genotypes selected from the previous study. The study was conducted with a completely randomized factorial design with two factors, namely EMS concentrations with 3 levels (0.1, 0.3 and 0.5%) and the soaking time periods consisted of 5 levels (0, 30, 60, 120 and 180 minutes) for each genotype. The treatment consisted of 5 replicates, and each replicate comprised 5 bottles with calluses. Observations were made after the calluses were 1 week old which included the number of surviving calluses. The calluses with the size of about 0.5x0.5x0.5 cm^3 were soaked in an EMS solution that had been sterilized using Millipore 0.2-0.4 µ with concentrations of 0.1, 0.3 and 0.5% (v / v) with soaking time periods of 0, 30, 60, 120 and 180 minutes using the method of Sakin *et al.* (2002), which had been modified. After EMS treatment, calluses were washed using sterile distilled water three times and dried in a petri dish that had been filled with filter paper. The calluses were grown in MS media + 2.4 D 3ml / l, and they were incubated in the dark for 1 week. Radiosensitivity concentration could be calculated with LC_{50} approach, that is, the EMS concentration that caused 50% explants to die. Observations were focused on the explants that were still alive at LC_{50} concentration. The determination of LC_{50} was conducted using a software of Probit Analysis.

In Vitro Selection of Callus at Temperatures of 27°C - 35°C.

This study is a continuation of the previous one, that is, the selection of callus based on 2 genotypes selected from EMS concentrations and soaking time periods that produced LC_{50} at temperature treatments. The optimal callus age used in vitro selection was about 4 weeks (Hsissou & Bouharmont, 1994). The explants were incubated in the dark for 2 weeks. The study was conducted with a completely randomized factorial design with a single factor, namely the temperature with 5 different degrees (27, 29, 31, 33, 35°C) for each genotype. The treatment consisted of 5 replicates, and each replicate comprised 1 bottle with 5 calluses. The data were analyzed for their variants using SAS 9.1, and if there was a significant difference, the analysis was continued using DMRT at a level of 5%. Observations were made on the number of living calluses. Selection criteria were based on the ability of the calluses to survive in the selection of temperature conditions. The calluses which were able to survive during the selection periods were categorized as resistant explants. On the contrary, the calluses that could not survive or die during the selection periods were considered as the explants which were not resistant to temperature selections. The resistant calluses were considered as putative mutants.

Calus regeneration as in vitro selection result

The study was conducted to obtain an appropriate media formulation to regenerate two (2) callus genotypes of putative mutants as the selection result in the previous study. The study used a completely randomized design with a single factor, namely media formulation with 7 levels as follows: 1) MS + without ZPT (control) (RG0). 2) MS + Kinetin 2 mg/l + Tyrosin 0.05 gr/l + Sorbitol 6% + Sucrosa 3% (RG1). 3) MS + BA 0.1 ml/l + Kinetin 2 mg/l + Tyrosin 0.05 gr/l + Sorbitol 6% + Sucrosa 3% (RG2). 4) MS + BA 0.5 ml/l + Kinetin 1 mg/l + Tyrosin 0.05 gr/l + Sorbitol 6% + Sucrosa 3% (RG3). 5) MS + Kinetin 2 mg/l + Tyrosin 0.05 gr/l + Sorbitol 6% + without Sucrosa 3% (RG4). 6) MS + BA 0,1 ml/l + Kinetin 2 mg/l + Tyrosin 0.05 gr/l + Sorbitol 6% + without Sucrosa 3% (RG5).7) MS + BA 0.5 ml/l + Kinetin 1 mg/l + Tyrosin 0.05 gr/l + Sorbitol 6% + without Sucrosa 3% (RG6). Each treatment consisted of five (5) replicates, and each replicate comprised 1 bottle with five (5) calluses. The bottle was incubated in a bright room with fluorescent light irradiation for 16 hours. The data were analyzed using SAS 9.1 program, and if there was a significant difference, the analysis was continued using Duncan Multiple Range Test (DMRT) at a test level of 5%. The observations conducted were to find out the percentage of explants forming buds and roots. All observations were done every month to form plantlets.

Results and discussion

The best formation induction of embryogenic callus

Immature embryos which were isolated from wheat seeds could form callus on MS media + 3 mg / l 2.4-D. This occurred since auxin with the powerful activity like 2.4-D was commonly used for inducing embryogenic callus (Sellers *et al.* 1990). The concentration of 2.4-D used in this study was similar to the one conducted by Shah *et al.* 2003, that is, evaluating the most optimal ZPT concentration for the induction of wheat callus which was 2.4-D 3.5 mg / l and 3mg / l (Sharma *et al.* 2005). This study, which also demonstrated the Kohsar varieties with a media of 2.4-D 3 mg / l, was able to induce callus by 83.3%, while the variety of Khiber-87 with a media of 2.4-D 3.5 mg / l was able to induce callus by 77.7% (Noor *et al.* 2009).

Table 1. Result of probit analysis of LC_{20}-LC_{50} in the varieties of Selayar and Dewata.

Genotype	Selayar			Dewata		
Concentration	0.10%	0.30%	0.50%	0.10%	0.30%	0.50%
Probability	Time Estimate	Time Estimate	Time Estimate	Time Estimate	Time Estimate	Time Estimate
.500 (LC_{50})	131.985	111.551	33.686	124.589	96.962	37.580
.550	121.961	97.472	29.283	117.363	87.436	32.674
.600	111.775	83.167	24.808	110.020	77.756	27.688
.650	101.248	68.381	20.184	102.430	67.752	22.535
.700	90.154	52.799	15.310	94.432	57.208	17.104
.750	78.181	35.984	10.051	85.801	45.830	11.244
.800 (LC_{20})	64.849	17.259	4.194	76.190	33.160	4.718

On the average, callus formation occured on day 7 after incubation, which is consistent with the research carried out by Satyavathi *et al.* (2004), who used a young embryo with the varieties of Ben, Munich, Lebsock and Maier on different callus induction media, which took between 3-7 days after induction. Meanehile, the use of mature embryos on a concentration media of 2.4-D 2mg / l + various sorbitol concentrations produced callus formation time between 3-7 hsi (Hasan *et al.* 2009). The results of the percentage analysis of proembryo callus formation in several varieties of immature embryo explants varied widely. Callus formation could produce proembrionic callus on all the six varieties.

The results showed that the lowest callus formation (36%) was on HP1744 to the highest (76%) was on Dewata (Figure 1A). This is consistent with the study of callus induction from the wheat of Durum variety and callus regeneration from local wheat (Sarker and Biswas 2002), which concluded that the explants from immature embryos have the highest percentage of callus formation and shoot regeneration compared to the explants from mature embryo, since the callus derived from immature embryos was potentially used for shoot regeneration to form a complete plant. Based on the percentage result, two varieties that had the best percentage of proembrionic callus growth were chosen: Dewata (76%) and Selayar (70%) (Figure 1A). The success of callus formation in each variety vary due to the differences in explant, genotype, genotype origin and adaptation to the target environment (Sarker and Biswas 2002). In addition, the differences in the ability to form callus in Dewata with the others was because Dewata has been adapted in Indonesia. Dewata and Selayar had the highest percentage of callus formation of the six varieties because the two varieties were already released in Indonesia in 2003-2004.

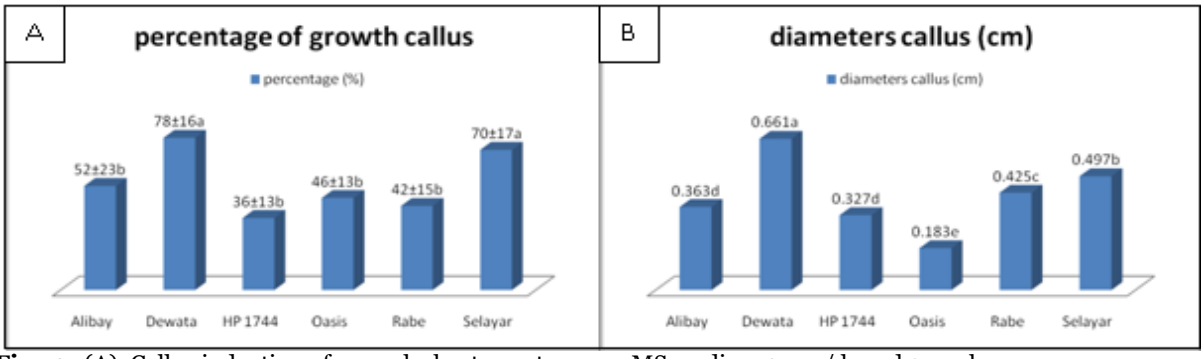

Fig. 1. (A). Callus induction of several wheat genotypes on MS media + 3 mg / l aged 2 weeks.
(B). Callus diameters of several wheat genotypes on MS media + 3 mg / l aged 2 weeks.

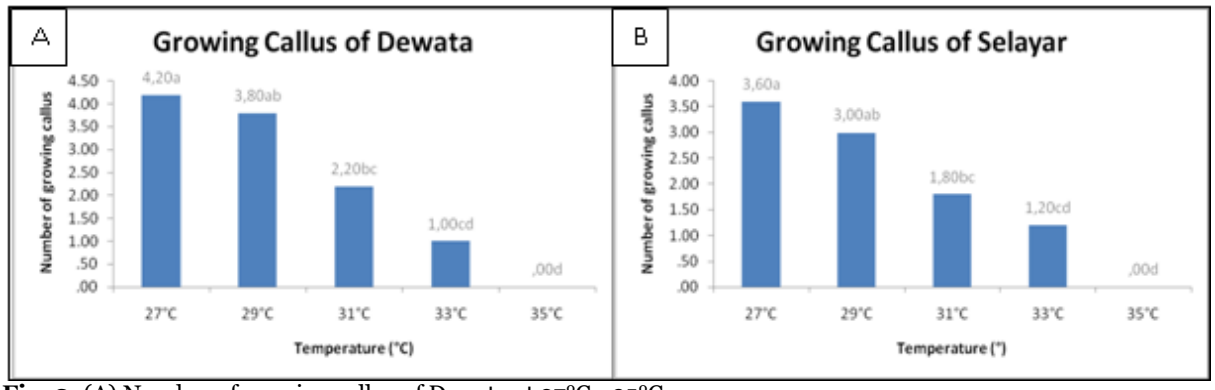

Fig. 2. (A) Number of growing callus of Dewata at 27°C - 35°C.
(B) Number of growing callus of Selayar at 27°C- 35°C.

The analysis result showed that the observation diameters of wheat calluses on all varieties had a significant effect on the media of MS +2.4-D 3mg / l on the observations of 2 msi. The diversity of callus diameters showed the differences in each wheat variety from the lowest (0.18 cm) in Oasis until the highest (0.66 cm) in Dewata (Figure 1B). The diference in callus diameter sizes occurred because the ability of each variety was different in forming amorphous cells which were formed from the initial tissue cells that divided continuously, so that the larger the diameter of calluses, the higher the activity and the number of initial tissue cells that divided. This diversity produced two varieties with high callus diameters of 0.66 cm (Dewata) and 0.50 cm (Selayar) (Figure 1B).

Induction of mutation in embryogenic callus using EMS

The embryogenic calluses of wheat with the varieties of Dewata and Selayar, which were induced using EMS, had produced optimum Lethal Concentration 50 (LC_{50}). LC_{50} is the value in which 50% of the calluses with EMS treatment could survive and thrive, and could be regenerated. The sensitivity level of a tissue to EMS concentration can be identified through radiosensitiviy. A proper dosage based on radiosensitivity is very important to determine the success in obtaining desired mutant variants. Radiosensitivity could be determined through the response of wheat calluses that could survive induced by EMS in LC_{20}-LC_{50}.

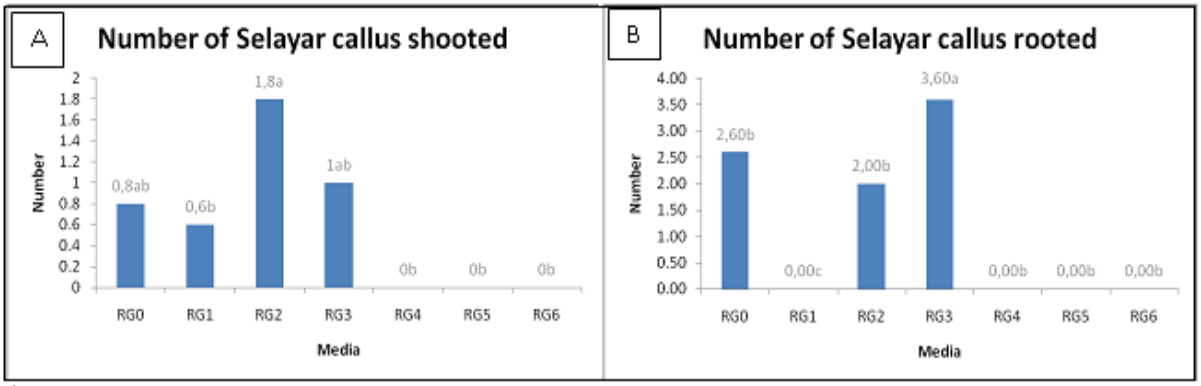

Fig. 3. (A) Number of Selayar callus shoots in various media.
(B) Number of Selayar rooted calluses in various media.

The observation result of callus growth of Selayar and Dewata varieties showed that the higher the EMS concentration used in wheat callus soaking, the less the time needed would be (Table 1). Dewata variety with the lowest concentration of 0.1% took 76.190 minutes, and the highest concentration of 0.5% took 4.178 minutes. In the meantime, in Selayar, the lowest concentration of 0.1% took 64.849 minutes, and the highest concentration of 0.5% took 4,194 minutes. (Table 2). Damaged calluses will reduce their ability to regenerate and cause cells to die, making them unable to regenerate (Biswas *et al.*, 2002). Increased EMS concentration and soaking time usually inhibit the growth of cells and eventually lead to cell death. According to the research conducted by Dhanavel *et al.*, (2008) and Biswa *et al.*, (2002), EMS application could inhibit cell division, resulting in the death of plant cells due to direct chemical mutagens, especially through toxic immersion, making the cells unable to proliferate to form shoots.

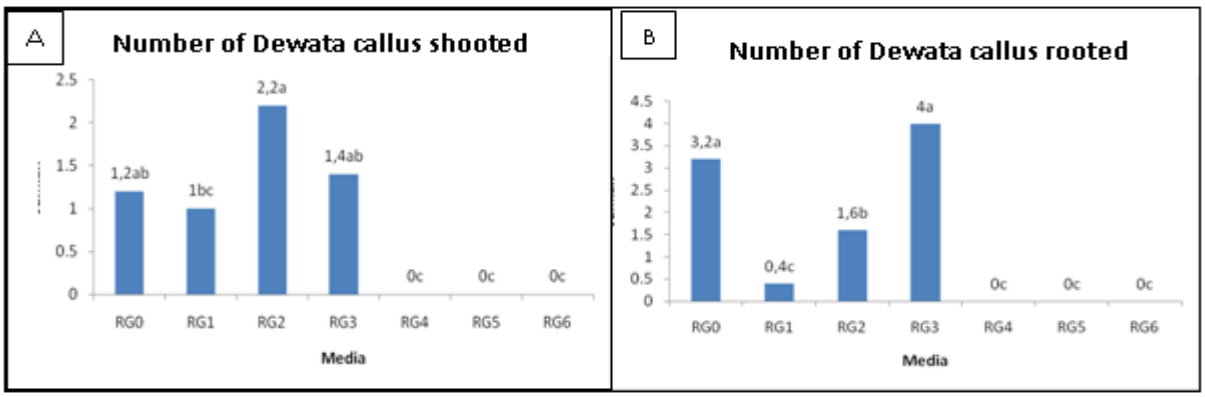

Fig. 4. (A) Number of Dewata callus shoots in various media.
(B) Number of Dewata rooted calluses in various media.

Both the test varieties--Dewata and Selayar--showed different responses to EMS treatments and soaking time periods. The EMS concentration required to cause diversity for each plant varied, depending on the genotype and explant type used, for example, in rice (grain) of MR219 cultivar, the EMS concentrations for LC_{25} and LC_{50} were 0.25 and 0.50% (Talebi *et al*., 2012); in wheat plants (seed) of B936 variety, the EMS concentration was 0.7% (Nidou *et al*., 2013). In this study, in Dewata variety, the EMS concentration used was 0.3% with a 30 minute soaking time, whereas in Selayar variety the optimal concentration of EMS was lower, that is, 0.1% with a longer soaking time, that is, 60 minutes.

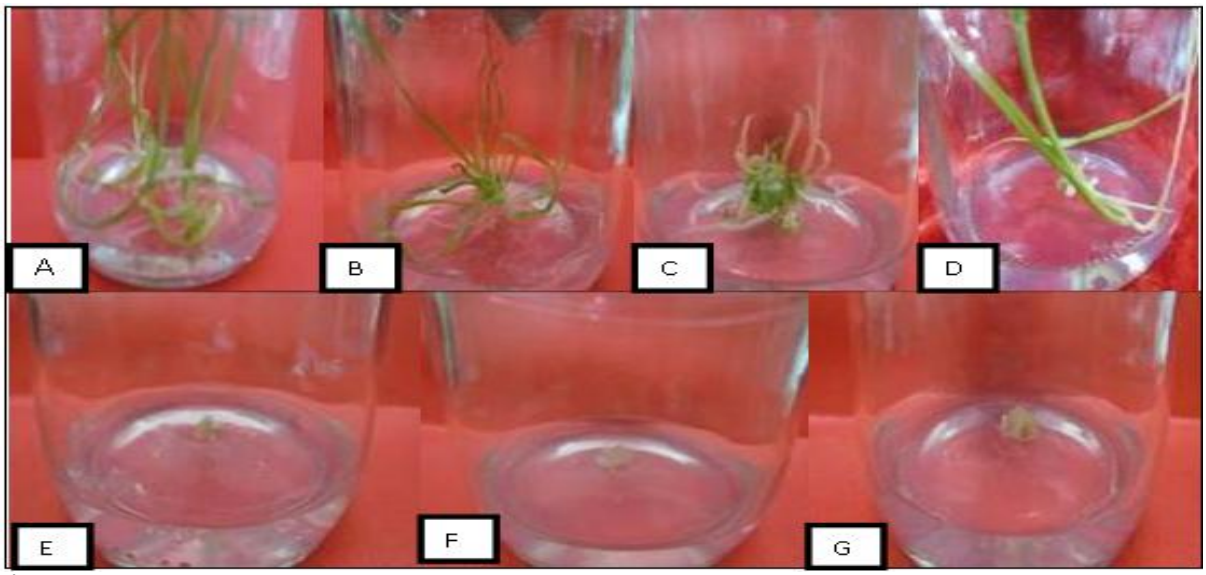

Fig. 5. Growth Selayar its regeneration into plantlets on a variety of media. A) MS + without PGR (control) (RG0). B) MS + Kinetin 2 mg / l + tyrosine 0:05 gr / l + Sorbitol + Sucrose 6% 3% (RG1). C) MS + BA 0.1 ml / l + Kinetin 2 mg / l + tyrosine 0:05 gr / l + Sorbitol + Sucrose 6% 3% (RG2). D) MS + BA 0.5 ml / l + Kinetin 1 mg / l + tyrosine 0:05 gr / l + Sorbitol + Sucrose 6% 3% (RG3). E) MS + Kinetin 2 mg / l + tyrosine 0:05 gr / l + Sorbitol6% + without Sucrose (RG4). F) MS + BA 0.1 ml / l + Kinetin 2 mg / l + tyrosine 0:05 gr / l + Sorbitol 6% + without sucrose (RG5) .G) MS + BA 0.5 ml / l + Kinetin 1 mg / l + 0:05 tyrosine g / l + Sorbitol 6% + without sucrose (RG6).

The rate of callus growth reduction of Dewata variety was 20% (LC_{20}) obtained at a concentration of 0.3% for ± 30 minutes (33.160). The rate of callus growth reduction of Selayar variety was 20% (LC_{20}) obtained at a concentration of 0.1% for ± 60 minutes (64.849) (Table 1).

The result of probit analysis indicated that the reduction of wheat callus growth of Dewata variety by 20-50% (LC_{20}-LC_{50}) was in the range of 0.3% for 33.180-96.962 minutes. Meanwhile, in Selayar variety the reduction of wheat callus growth was in the range of 0.1% for 64.849-131.985 (Table 1). The ranges, theoretically, were the LC that could lead to a high diversity of mutants due to the mutation in wheat callus tested. The time range required in LC obtained from this study was very low compared with the study by Sakin (2002). Sakin and Yildirim (2004), who produced a high genetic diversity of wheat and got a high yielding variety that ranged from 0.1 to 0.3 with 8 hours soaking time in the wheat seeds of s Gediz-75 variety. The concentration range obtained in Dewata variety was 0.3% for 30 minutes, and Selayar was 0.1% for 60 minutes, where the concentration and the time were the ranges right to produce mutant diversity (Table 1).

In vitro selection of callus at temperatures of 27°C - 35°C.

Genetic diversity generated by mutation induction

with EMS is in random, and therefore it is necessary to have in vitro selection techniques to obtain the desired change. The calluses of Dewata and Selayar varieties which were selected using a temperature of 35°C experienced a change and inhibition in their proliferation as well as cell death, so that the selected calluses were blackish brown and could not survive. This indicated that the temperature of 35°C created the inhibition of callus growth. The higher temperature selection, the greater the inhibition would be. The calluses which were selected at a temperature of 27°C had the highest number of callus growth, namely 4.2 and 3.6 (Figure 2). The calluses which were resistant to selection temperature could grow and produce shoots when planted in the regeneration media.

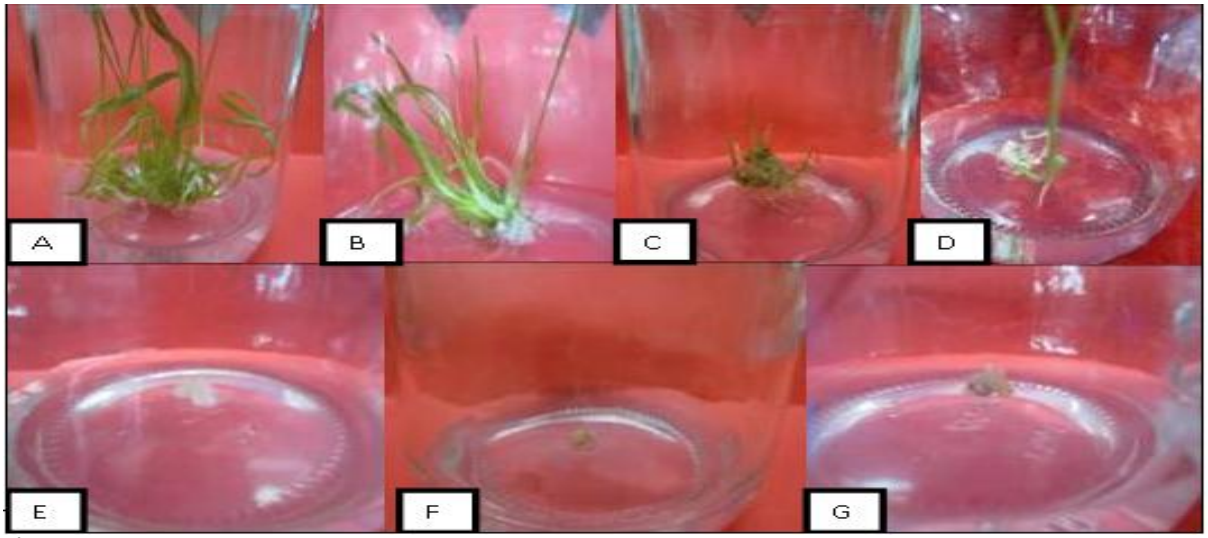

Fig. 6. Growth Selayar its regeneration into plantlets on a variety of media. A) MS + without PGR (control) (RG0). B) MS + Kinetin 2 mg / l + tyrosine 0:05 gr / l + Sorbitol + Sucrose 6% 3% (RG1). C) MS + BA 0.1 ml / l + Kinetin 2 mg / l + tyrosine 0:05 gr / l + Sorbitol + Sucrose 6% 3% (RG2). D) MS + BA 0.5 ml / l + Kinetin 1 mg / l + tyrosine 0:05 gr / l + Sorbitol + Sucrose 6% 3% (RG3). E) MS + Kinetin 2 mg / l + tyrosine 0:05 gr / l + Sorbitol6% + without Sucrose (RG4). F) MS + BA 0.1 ml / l + Kinetin 2 mg / l + tyrosine 0:05 gr / l + Sorbitol 6% + without sucrose (RG5) .G) MS + BA 0.5 ml / l + Kinetin 1 mg / l + 0:05 tyrosine g / l + Sorbitol 6% + without sucrose (RG6).

According to Svabova & Lebeda (2005), the method of utilizing selection agent which is effective in vitro can help improve the desired plant characteristics, for example, the use of temperature to get heat resistant plants. Heat resistant plants are plants that are able to maintain the integrity of plant membranes in heat stress conditions. That is why the plants are called mutants. Mutant plant is the plant that will be selected to be regenerated on regeneration media. Dewata variety has adaptive tolerance at a temperature of 27°C, and the higher the temperature, the less tolerance the plants have. The decreased adaptation tolerance in the plants is the result of the stress caused by high temperatures. According to Sung *et al.* (2003), the magnitude of stresses caused by temperature varies, depending on the temperature intensity, the length of stress period and the rate of temperature change. The values of callus growth at temperatures 27, 29, 31, 33 and 35°C were respectively 4.2, 3.8, 2.2, 0 (Dewata) and 3.6, 3, 1.8, 1.2, 0 (Selayar) (Figur 2). According to Wiyono 1980, for its growth, wheat required temperatures around 15-25°C and could not grow in warm areas and high temperatures. 1°C increase only will inhibit wheat growth.

Callus regeneration as a result of in vitro selection.

To obtain a lot of shoots is very difficult in wheat callus regeneration; one callus only produces 1-2 shoots. This is consistent with the statement of

Hassan *et al.* (2009) saying that the tissue culture of monocotyledonous plants of gramine family is relatively more difficult to regenerate compared to dicotyledonous plants. Shah *et al.* (2009) reported that monocot explants were difficult to regenerate because they do not have cambium. The result of variance analysis showed that the media formulation had a significant effect on shoot formation, both in Selayar and Dewata. The varieties of Dewata and Selayar had the highest number of shoots produced on RG2 media (MS + BA0.1ml / l + kinetin 2mg / l + Tyrosin 0.05gr / l + Sorbitol 6% + Sugar 3%), that is, 1.8 and 2.2. The next was followed by RG3 media 1 and 1.4, RG0 media 0.8 and 1.2, RG1 media 0.6 and 1 (Figure 3A, 4A). RG2 media formulation was thought to have an optimum concentration for shoot formation. This study is line with the research result of Sarker and Biswas (2002) which stated that the optimal media formulation for wheat callus regeneration from immature embryo explants to shoot was MS media +0.5 mg / l BAP +0.5 mg / l Kinetin +25 mg / l tyrosine.

The Media of RG4, RG5 and RG6 did not produce shoots and roots on both varieties Dewata and Selayar (Fig. 5 and 6). On those media the color of calluses changed into white, and there were no signs of life until the age of 12 weeks. No sugar was added to the formulation of media RG4, RG5 and RG6. Sugar is a carbohydrate source for a plant so that it can grow and develop, and without sugar there would be no energy. The reseach result concluded that sugar is still needed in the media for plant growth. It was found that sugar (sucrose) is more easily absorbed by plants than alcohol sugar (sorbitol). The addition of sorbitol is also done so that shoots grow rapidly. According to George & Sheringtom (1984), sucrose is basically an important source of carbon used as a constituent of cells, such as for cell division, cell enlargement and cell differentiation.

In this study, the shoots started to grow in week 2 after the appearance of green spots in week 1. This study is in line with the report of Hassan *et al.* (2009) that stated that the wheat varieties called Inwafaq-2001, Inqilab-91 and Auqab-2002 could produce shoots in week 4 in MS media with the addition of sorbitol, whereas in the media without sorbitol they might take more than 4 weeks. The addition of sorbitol has the power to regenerate plants so that only tolerant plants can regenerate. Media to which sorbitol is added serves to increase plant regeneration and increase the efficiency of using of MS media. The addition of sorbitol in the regeneration medis is used to select plants that are tolerant and can adapt so that they can grow into complete plants ready for acclimatization. RG2 media could produce the highest number of shoots which could grow into complete plants. As indicated in the research of Sharma *et al.* 2005, the regeneration media to which 6% mannitol is added can produce shoots with a height of 5-10 cm; below and above the percentage, the shoots can grow 1-5 cm high. This indicated that the plants which are optimally tolerant use sorbitol or mannitol by 6%.

The most rooted calluses were produced by the varieties of Dewata and Selayar on RG3 media (MS + BA0, 5ml / l Kinetin + 1mg / l + tyrosine 0.05 g / l + Sorbitol 6% + Sucrose 3%), that is, 3.6 and 4.0 (Figure 3B and 4B). The next was followed by media RG0, namely 2.0 and 3.2; after that, media RG2 with a value of 1.6 (Selayar) and 2.0 (Dewata); and finally, media RG1 with a value of 0.4 (Dewata). Meanwhile, in Selayar variety the media RG1, RG4, RG5 and RG6 had no roots. In Dewata variety, only 3 media had no roots: RG4, RG5 and RG6. RG3 media had a tendency to form calluses with many roots (rhizogenic callus) and it formed a small number of shoots, probably because the interaction between genotype and ZPT which were added to media RG3. According to Purnamaningsih & Mariska (2005) rhizogenic callus is the callus that forms roots faster than shoots. Rhizogenesis usually occurs in a combination of media treatment that contains higher auxin than cytokinin (George & Sherington, 1984). In this case, it did not go that way, although there was no addition of auxin to RG3 media, callus would first form roots, and the longer, the more. This is presumably because the endogenous auxin hormone role in wheat plant is very high so as to induce a number of roots which are

greatly formed (Fig. 3B and 4B).

Conclusion

Embryogenic callus which gave the highest response was the one generated on the varieties of Dewata and Selayar since they have been well adapted in Indonesia. Increasing EMS dose and soaking time in embryogenic callus inhibited the growth of callus. The higher EMS dose applied, the less calluses can live. In vitro selection that produced the highest callus was at a temperature of 27°C. The best media formulation for regeneration was the media of RG2 (MS + BA0.1ml / l + kinetin 2mg / l + tyrosine 0.05 gr / l + Sorbitol 6% + Sucrosa 3%), that is, 2.2 (Dewata) and 1.8 (Selayar).

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