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Variation induction of *Glycine max* through low dose gamma irradiation produces genetic and physiological alteration as source of tolerant variants in waterlogging conditions

TRIONO BAGUS SAPUTRO[1,♥], WIRDHATUL MUSLIHATIN[1], DWI KUSUMA WAHYUNI[2], TUTIK NURHIDAYATI[1], FILA OXI WARDHANI[1], ELENA ROSALIA[1]

[1]Department of Biology, Faculty of Sciences, Institut Teknologi Sepuluh Nopember. Jl. Arif Rahman Hakim, Keputih, Sukolilo, Surabaya 60111, East Java, Indonesia. Tel.: +62-31-5963857, Fax.: +62-31-5963857, ♥email: trionobsaputro@bio.its.ac.id, trionobsaputro@gmail.com
[2]Department of Biology, Faculty of Science and Technology, Universitas Airlangga. Kampus C, Mulyorejo, Surabaya 60115, East Java, Indonesia

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Abstract. *Saputro TB, Muslihatin W, Wahyuni DK, Nurhidayati T, Wardhani FO, Rosalia E. 2019. Variation induction of* Glycine max *through low dose gamma irradiation produces genetic and physiological alteration as source of tolerant variants in waterlogging conditions. Biodiversitas 20: 3299-3308.* Soybean (*Glycine max* L.) is the main food commodity that contains high proteins, oils, and carbohydrates. Waterlogging is considered as the dominant factor and major constraint responsible for the decrements of soybean production in Indonesia. The development of promising *G.max* that tolerates waterlogging is essentially needed. In this study, induction of variation was conducted by gamma rays irradiation with doses of 25Gy, 50Gy, 75Gy, and 100Gy. The mutant or variants lines were then selected under waterlogging conditions with 100%, 150%, 200%, and 250% of field capacity. The results showed that, in 250% waterlogging condition, plants irradiated with 25Gy shows the best performance in amount of roots, adventitious roots and number of pods parameters, while plants irradiated with 50Gy have the highest growth indicated by amount of root nodules, plant heights, dry weights, leaf area, and chlorophyll contents. The genotypes then were assayed by the Inter Simple Sequence Repeats (ISSR) to confirm that the mutants differ compared to its wild type. Out of 10 ISSR primers, seven primers showed polymorphic patterns. The best primer to differentiate the mutant lines and its wild type is primer ISSR1 [(AC)8G] that able to generate the highest level of polymorphism with 44.0%. The comparison of protein profiles among the mutant lines showed that proteins with molecular weight 53,78 KDa; 43,12 KDa; and 20,62 Kda are all overexpressed for plants irradiated at 25Gy and treated with 250% waterlogging stress. All those three proteins are predicted as 1-amino cyclopropane-1-carboxylate synthase (ACS), Alcohol-dehydrogenase (ADH), and Superoxide dismutase (SOD) respectively.

Keywords: ACS, ADH, gamma rays, *Glycine max*, ISSR, MI, PIC, SDS-PAGE, SOD, waterlogging

INTRODUCTION

Soybean (*Glycine max* L.) has been widely cultivated in Indonesia, and one of its varieties that is widely cultivated is Anjasmoro, which has a considerable yield of 2.03-2.25 tons/ha (Adie and Krisnawati 2007). High protein and fat contents make soybean as an important raw material in various food industries (Stein et al. 2008; El-Shemy 2011). Soybean consumption in Indonesia tends to increase, but this condition is not linear to the production rate, which indicates a decline. The decline is mainly caused by sensitivity to abiotic stress, including waterlogging (Wang et al. 2017). Excess water conditions can cause serious problems in plant growth and productivity. According to Pauw et al. (2010), waterlogging conditions can reduce crop yields by 25%. Waterlogging stress causes plants to be in anaerobic conditions, which activates the glycolysis pathway and fermentation (Setter et al. 2003). Waterlogging causes a decrease in photosynthesis and leaf expansion, little gas exchange, and results in a low growth rate as well as productivity (Morgan et al. 2004). Other effects of waterlogging stress are the small number of plants' leaves and the yellowing of leaves (Thomas and Sodek 2005). At the molecular level, stress causes excessive fermentation, Reactive Oxygen Species (ROS) production which leads plants to death, increasing levels of plant glycolysis, and rooting system changes (Hashiguchi et al. 2009).

Those problems can be solved by improving plant ability to survive within the stress conditions. Nowadays, there are still very few sources of genes tolerant of waterlogging in soybean, so it is necessary to induce genetic diversity over species. An induction of variation is needed to trigger mutations in order to produce new variants that can withstand waterlogging conditions. Physical mutagen, among others, is by using UV light (UV-A, UV-B, or UV-C) (Castronuovo et al. 2014), X-rays (Maghuly et al. 2017), or gamma-ray irradiation. Gamma rays is a popular method in inducing diversity and crop improvement (Hanafiah et al. 2010). Irradiation has been proven to increase soybean production (Mudibu et al. 2011; Aminah et al. 2015). In the previous studies, various doses of irradiation had been applied to improve the agronomical characteristics of plants, including orchids (Bondada and Oosterhuis 2003) and canna (Celik et al. 2014). Low irradiation doses are also effective in increasing resistance

of food crops under certain stress conditions such as wheat and soybean with drought stress (Das et al. 2002, Aminah 2015), and rice with saline stress (Chaerle et al. 2001).

All the variants produced by irradiation are random which means needs to be selected to obtain the target traits. Selection is conducted to obtain plants with desirable characteristics, including the increase in tolerance to waterlogging stress. The level of tolerance to waterlogging stress of soybean can be observed through several growth parameters. In addition, it is also necessary to observe the genetic diversity of waterlogging-tolerant plants due to irradiation. Genetic diversity analysis methods have progressed very well starting from morphological, biochemical and molecular analysis. At present, molecular markers are the most appropriate marker to provide an overview of genetic diversity in a species. Molecular markers provide information at DNA level that is able to overcome the morphological observation bias due to environmental differences and excellently assess polymorphism in plant genome to observe genetic diversity. Several molecular marker techniques can be applied for plant genetic diversity analysis, included ISSR (Inter Simple Sequence Repeats). ISSR markers are considered as a simple and quick method, it amplified the microsatellite regions of DNA sequences, and does not require any information about gene sequence (Ansari et al. 2012).

In this study, mutant lines of soybean were developed through gamma irradiation and then selected for their tolerance to waterlogging stress. The genetic diversity of the mutant lines and their respective wild type were analyzed using ISSR markers. In addition, the protein profiles of the lines were also analyzed using SDS-PAGE.to prove that the mutant lines are different from the initial line in responding waterlogging stress.,

MATERIALS AND METHODS

Seed irradiation

This study used Anjasmoro variety of seeds obtained from the Legumes and Tuber Crop Research Institute (BALITKABI), Malang, Indonesia. The seeds were surface sterilized with 70% ethanol for 2 minutes and 1% NaOCl (Sodium hypochlorite) for 5 minutes. Gamma-ray exposure was then carried out at five levels of treatment, i.e. 25 Gy, 50 Gy, 75 Gy, and 100 Gy, on 350 seeds for each irradiation dose conducted at the National Atomic Technology Agency (BATAN).

Seeds germination

Unirradiated (non-irradiated) and irradiated seeds were soaked for 6 hours using aquadest and then transferred to the germination medium. Materials used as germination media including soil, organic fertilizer, charcoal, husk, and water put into portray. The seeds were grown in portray containing the planting medium and then sown until 2 cotyledon leaves appeared, this stage named Vegetative Emergence (VE). The seed of soybean needs 5-10 days to reach this stage. In this study, we used 7 days. The seedlings were subsequently transferred in a polybag containing 2 kg of soil and 0.5 kg of organic fertilizer for 14 days or after the first two trifoliolate leaf nodes appeared, this stage named Vegetative 2 (V2).In the next step, plants with similar height and stage on each irradiated dose were treated for waterlogging stress conditions.

Waterlogging stress condition

Prior to waterlogging treatment, the field capacity was measured following the method employed by Fatimah and Saputro (2016). The measurement of field capacity was used to decide the additional water content to simulate waterlogging stress. Waterlogging was carried out for 14 days at five levels (0%, 100%, 150%, 200%, and 250%) for all irradiated seeds treatment (0 Gy, 25 Gy, 50 Gy, 75 Gy, and 100 Gy). Waterlogging stress treatment was applied by giving water daily to each polybag according to the predetermined concentrations based on the results of the field capacity calculation (Foth 1984). A hundred percent of waterlogging is given by 100% of field capacity, while 150% of waterlogging is 150% of field capacity and the same for other concentrations. The experiment was performed by the usage of two sets of plant with three replication in each set. The first set was used for destructive assessment methods and observed after 14 days in waterlogging stress treatment. The parameters such as number of roots, number of adventitious roots, leaf area, plant dry weight, and chlorophyll content were observed using the first set. Leaves for DNA sample and protein samples were also. The second set was treated 14 days in waterlogging condition and grown normally using 100% of Field capacity after the treatment. This set was harvested at 85 days as instruction in variety description of Anjasmoro variety or can be described that plants are in reproductive stage 6 (R6). Furthermore, root nodules, plant height, and number of pods were observed at this stage.

Agronomic parameters

The amount of root and adventitious root were carried out after 14 days of waterlogging treatment by counting the number of root and root nodules formed at the stem base at each treatment. Subsequently, after counting those two parameters, all parts of plants were dried using oven at 80^{o}C for three days then weighed using analytical balance to obtain dry weight data. Leaf area was estimated using gravimetric method. Leaf samples used were taken from the same node at each plant. Leaf was traced on the width of a piece of paper and cutted which considered as replica. Leaf area is calculated based on the ratio of the weight of the leaf replica to the total weight of the paper. The calculation formula is as follow (Sitompul 1995):

$$La = (Wr \times Pa)/Wt$$

Where:
La = leaf area (cm^2);
Wr = weight of replica (g);
Wt = weight of total paper (g);
Pa = total paper area (cm^2).

Measurement of plant height was carried out after the harvesting (plant height). Soybean height is measured from the base of the stem to the tip of the leaf (shoots). The amounts of root nodules were carried out after 85 days (R6) by counting the number of root and root nodules at each treatment. Observation of the pods was carried out after 85 days by counting the number of pods produced on the plant.

Chlorophyll content

Measurement of chlorophyll content was performed in each waterlogging concentration. Soybean leaves were weighed for 100 milligrams with an analytical balance BOECO Germany BBI-31. Chlorophyll content was measured by employing Wintermans and Mots (1965) method using UV-Vis spectrophotometer with wavelengths of 649 nm and 665 nm. The total chlorophyll was calculated as = [20.0 x A649 + 6.10 x A665].

DNA Extraction and ISSR analysis

The leaves from treated and non treated plants were subjected to DNA isolation. DNA isolation was performed using the CTAB method (cetyl trimethyl ammonium bromide) based on (Saputro et al. 2018). DNA amplification reaction was conducted using PCR with the master mix reagent from Bioline. Ten ISSR primers were used in this study, based on several literatures (Mudibu et al. 2011; Kumar et al. 2009; Husni 2010; Alamri 2014) namely ISSR-1 (AC)8G; ISSR-2 (TCC)5GC; ISSR-3 (GA)8T; ISSR-4 (GT)8TC; ISSR-5 (AG) 8T; ISSR-6 (CA)6GG; ISSR-7 (CT)8GC; ISSR-8 (GT) 6CC; ISSR-9 (CA)6AG; AND ISSR-10 (CAC) 3GC. The PCR process was begun with the initial denaturation stage at 95°C for 5 minutes. PCR was conducted at the initial denaturation at 95°C for 60 seconds, annealing with temperature according to the primers for 45 seconds, and extension at 72°C for 60 seconds, for 30 cycles. The PCR process ended with a final extension at 72°C for 5 minutes (Martasari, 2012). PCR product was then separated with 1% agarose gel by electrophoresis technique. Agarose gel was prepared by weighed 0.5 grams of agarose powder and added with 50 ml of 0,5x Tris Base EDTA (TBE) and boiled. Subsequently, after the temperature of solution drops to 40-50°C, 1 µL of gel red stain (dye for nucleic acid) was added and put on template. Electrophoresis system was conducted at 100 volt, 50 minutes. The separated fragments were visualized by Biostep UV Light Transilluminator. PIC (Polymorphism Information Content) in each primer was calculated based on Smith et al (1997) by the following formula:

$$PIC_i = 1 - \sum_{j=1}^{n} P_{ij}^2$$

EMR (Effective Multiplex Ratio) was calculated by using the formula α x β, in which α was the average number of amplified fragments on specific markers, while β was obtained from the calculation of the number of locus polymorphisms (PB) and the number of monomorphism loci (MB); β = PB / (PB + MB). MI (Marker Index) was calculated to obtain capacity characterization in each primer to detect locus of polymorphism, which was calculated using the formula MI = EMR x PIC (Varshney 2015).

Protein profile analysis

Protein was isolated from soybean leaves after 14 days of the waterlogging treatment. Samples were taken from the control and 250% waterlogging treatment for each irradiation dose. The method of isolation, measurement of protein concentration and electrophoresis referred to the research by Saputro et al (2018). In this study, protein ladder of 7µL (spectra multicolor broad range protein ladder) was used and added with 10 µL of sample buffer in sterile microtube. Measurement of protein molecular weight was calculated using ImageJ software. The distance of the bands was used as the ordinate of the curve (x-axis). The abscissa (y) axis of the curve was the log value of the marker band's previously known molecular weight. From the obtained abscissa and ordinate analysis, a Fitted Line plot curve was created. From the curve, the relationship between protein molecular weight and the distance traveled by the bands due to electrophoresis can be analyzed (Durrani et al. 2008).

Data collection and analysis

Data of ISSR marker and protein profile analysis were descriptively analyzed. Plant growth parameters and chlorophyll content data were statistically analyzed using one-way ANOVA at 5% significance level (α) to determine the effect of treatment on the observed parameters. Duncan Multiple Range Test (DMRT) at α 5% was carried out as Post-Hoc test using SPSS 16.0 program to compare the most effective waterlogging treatment.

RESULTS AND DISCUSSIONS

Morphology of irradiated soybean plants in waterlogging condition

The use of gamma rays which are the result of an unstable atomic nucleus is based on the characteristic of gamma rays that has the highest frequency and the shortest wavelength (10^{-12} m) among the spectra of other electromagnetic waves, so gamma rays have very high penetrating power (Sunardi et al. 2012). Irradiation is one of the techniques widely used in improving varieties (Elkalik et al. 2010). This method is effective since it can increase the genetic diversity of a variety. Genetic diversity due to irradiation treatment is caused by changes in genetic material of a plant, which includes changes in the composition of the nitrogenous base of the plant (Hanafiah et al. 2010). In this study, irradiation was given at 0 Gy (control), 25 Gy, 50 Gy, 75 Gy, and 100 Gy doses in soybean seeds of Anjasmoro variety. Characteristics of random mutations needed to be directed by using directional screening. Therefore, the irradiated plants were selected by exposure to waterlogging condition. The results showed that the waterlogging concentration had a significant effect on the parameters of root nodules, adventitious roots, wet weight, and dry weight of plants (Figure 1).

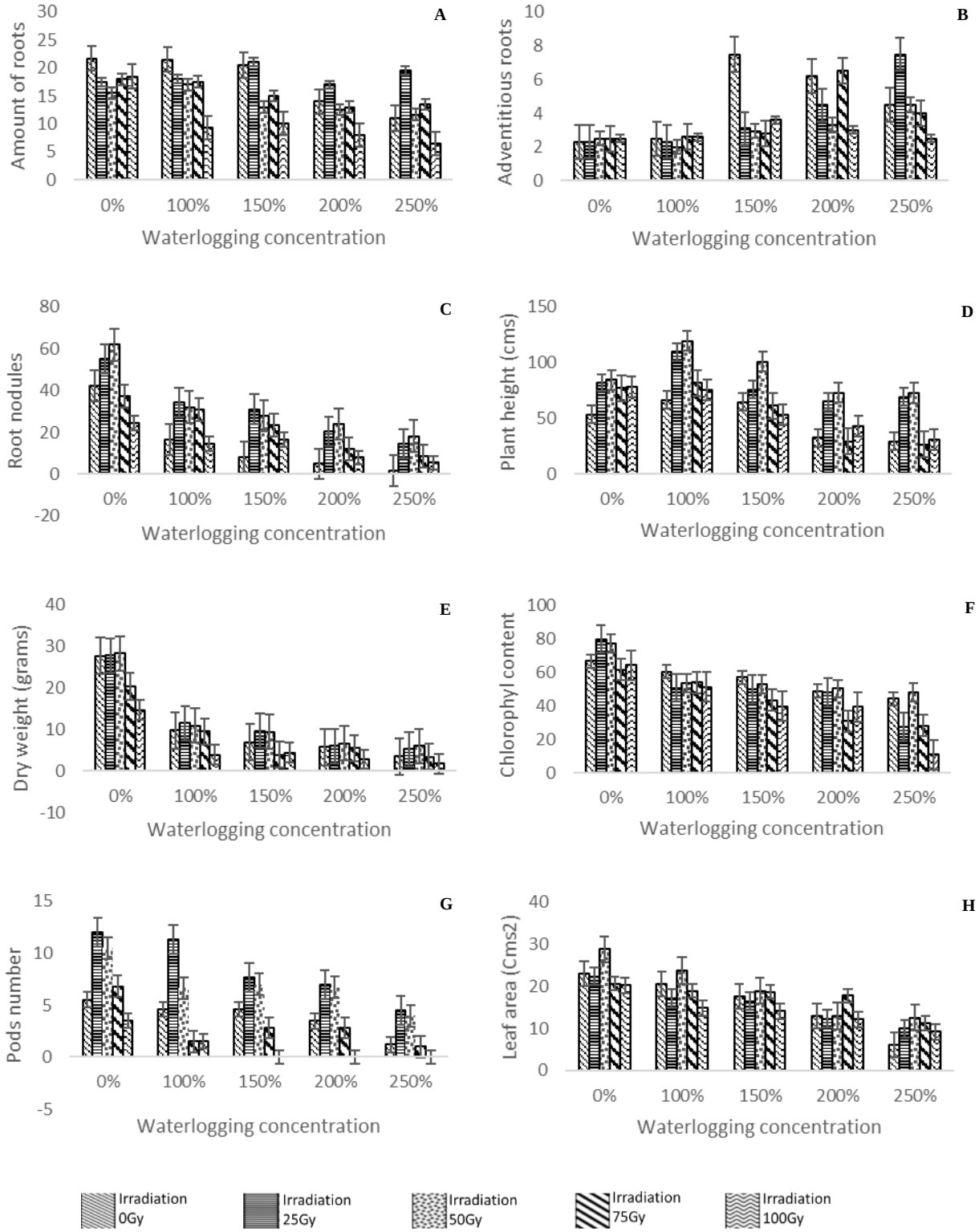

Figure 1. Performance of soybean after 14 days exposed to waterlogging. A. Amount of roots; B. Amount of adventitious roots; C. Amount of root nodules; D. Plant heights; E. Dry weights; F. Chlorophyll contents; G. Pods number; H. Leaf area

Number of roots, number of adventitious roots, and root nodules

Roots are one of the plant's main organs that function to absorb water and minerals. In waterlogging conditions, the concentration of oxygen in the soil will decrease (oxygen deficiency), resulting in a disturbance of plant metabolism which will eventually lead to root damage in the cylindrical part of vessels (n et al. 2011). Moreover, 0 Gy irradiation dose (control/wildtype) at 0% waterlogging (normal growth condition) showed the highest root number resulted in an average of 21.6 since the soybean seedlings were not waterlogged; at the waterlogging treatments, the root number of the wild type seedlings decreased to 21.4, 20.5, 14, and 11 roots at 100, 150, 200 and 250 % of waterlogging, respectively. This is in accordance with the result of Susilawati et al. (2011) which states that numerous roots will be damaged due to waterlogging conditions. On the other hand, among all irradiated as well as wildtype seeds the average number of soybean roots at 25 Gy irradiation considered as the highest root number at 150, 200 and 250% of waterlogging treatments.

As compensation for the decrease in the number of roots in waterlogging conditions, plants will tend to form adventitious roots. Adventitious roots are an indicator that plants experience waterlogging stress conditions and show that the plants are adaptable or tolerant to waterlogging (Jitsuyama et al. 2017). According to Sembiring et al. (2016), the adventitious roots serve to maintain the continuity of oxygen as well as mineral supply and replace the function of the main root, which cannot absorb maximum oxygen in waterlogging stress conditions. Adventitious root formation is influenced by the presence of waterlogged conditions in the planting area. This is in accordance with the statement of Suematsu et al. (2017) that in waterlogging stress conditions, soybean plants will grow adventitious and aerenchyma roots, which function to drain oxygen from the stem to the root. The highest number of adventitious roots at the waterlogging concentration of 250% was showed by the seeds irradiated at 25 Gy (Figure 1B).

Another parameter that can be used as the indicator of waterlogging is the presence of root nodules. Root nodules are very important for soybean plants because they require more nitrogen for the production process, which makes soybean seeds contain a lot of protein (Maekawa et al. 2011). Higher nitrogen concentration is obtained by using root nodules. Formed root nodules are the symbiosis results between Leguminosae species and Rhizobium bacteria by means of N_2 tethering in the atmosphere (Saito et al. 2014). Bacteria including nitrogen-fixing bacteria are *Bradirhizobium japonicum*, *B. elkanii*, and *B. lianigense* (Yoshiki et al. 2017). The root nodules function to fix nitrogen in the atmosphere and will later be added to the assimilation of inorganic nitrogen from the soil, so the productivity of soybean is more optimal (Ohyama et al. 2013).

Waterlogging stress conditions caused oxygen diffusion to be inhibited. This certainly affected the formation of nodules in soybean roots as the symbiosis between nitrogen-fixing bacteria and the roots of legume plants (Saito et al. 2014). The higher concentration of waterlogging stress caused the oxygen concentration to continue to decrease, causing bacterial respiration to be disrupted, so the formation of nodules decreased, conforming to the statement of Saito et al. (2014). It can be seen in Figure 1C that the number of root nodules decreased in the increasing waterlogging concentrations in all irradiation doses. The decrease in the average number of root nodules caused a decrease in nitrogen concentration that can be absorbed by the roots of normal soybean plants. The results of an ANOVA test showed that waterlogging significantly affected the average number of root nodules. Duncan's test results showed that the 50 Gy irradiation dose was the most effective dose in increasing the average number of root nodules (Figure 1C). Interestingly, all of irradiated seeds had higher number of root nodules at 150-250% of waterlogging compared to the initial line. According to Pinto et al. (2002), irradiation exposure increases root nodule formation by 60%.

Plant height

Irradiated soybean had better plant height than unirradiated soybean plants. Unirradiated soybean plants experienced a significant decrease in plant height at 200-250 % of waterlogging. Waterlogging stress causes a decrease in oxygen supply causing the metabolic activity of the plants inhibited and a reduction in plant height (Ferreira et al. 2007). The highest plant height resulted from the 50 Gy irradiation dose, followed by 25 Gy irradiation dose for all waterlogging treatments. The result was in accordance with Firsta and Saputro (2018) that found 25 Gy irradiation dose is the best dose to induce plant height. In addition, the treatment with low doses of gamma-ray irradiation can be used as a seed treatment that can improve germination and seed growth. Waterlogging stress treatment on the soybean plants irradiated at 75 Gy and 100 Gy indicated a shorter plant height compared to non-irradiated plants when exposed to the waterlogging concentrations of 150%. Furthermore, at 200 and 250%, the height of non-irradiated plants, 75 Gy and 100 Gy are not significantly different (Fig. 1D). Moreover, it can be stated that 75 and 100 Gy irradiation doses did not cause an improvement in the plant height when subjected to high waterlogging stress compared to low irradiation dose (25 and 50 Gy).

Dry weight

Soybean plants are sensitive to waterlogging stress that affects the germination period, the vegetative period, and the reproductive period (Githiri et al. 2006). The average plant dry weight depicted consistent decrease in all waterlogging treatments compared to 0% waterlogging concentration, at all irradiation doses (Figure 1 E). The results of ANOVA test showed that the irradiation dose and waterlogging concentration significantly affected the dry weight of the plants. Plants will alter their metabolism during anaerobic condition, including energy production by promoting the fermentation mechanism where plants could get only two ATP (Adenosine Triphosphate) per glucose molecule (Ashraf 2012). The ATP is organic molecules that provide energy to drive plant metabolism including the

synthesis of various proteins in photosynthetic system. In addition, Caudle and Maricle (2012) stated that *Phaseolus vulgaris* experience down-regulation of Photosystem II (PSII) during waterlogging treatment. PSII is an important protein complex that harvests energy from sunlight and transfers electron in light-dependent reaction. The hindering of metabolic activity is the main cause of reduction in plant productivity (Shaw et al. 2013) and exhibits low plant dry biomass. The lowest dry weight was observed in the irradiated seeds at 100 Gy stressed at 250% the waterlogging concentration. At 250 % of waterlogging concentration, the highest dry weight was produced by 50 Gy Irradiation seeds with 6.03 grams. A high irradiation dose might cause damage to the plant chromosome, resulting in disruption of the plant growth. According to Srivastava and Kumar (2011), the higher the dose of gamma radiation, the shorter the safflower plants compared to the control.

Leaf area and chlorophyll content

Another important plant parameter is the leaf, important plant organs, since it is the place for the occurrence of the photosynthesis and transpiration processes that determine plant growth. It was found that irradiation dose factor did not affect the leaf area of the soybean plants ($p = 0.249$) In terms of the waterlogging factor, it was found that waterlogging affected the leaf area of the plants. The increasing of waterlogging level generally reduced the leaf area either in the control or irradiated plants.

The highest leaf area was obtained at 50 Gy irradiation treatment, either in non-waterlogging (28.85 cm^2) or in the waterlogging condition of 250% (12.58 cm^2) (Figure 1 G). The lowest average leaf area was in non-irradiated plant that stresses the 250% waterlogging concentration. As the waterlogging level increased, the leaf area of irradiated soybean plants decreased. The decrease in leaf area is one symptom of the decrease in plant growth that can be observed when plants experience hypoxic or anoxic conditions. Abiotic stress, including waterlogging, can inhibit phytohormone synthesis. Cytokinin is one of phytohormone, derivatives of nitrogen adenine bases that can affect plant development processes, inducing differentiation in tissue culture, lateral shoot growth, leaf expansion, chloroplasts development, and delay in leaf aging (Hopkins et al. 2009).

Chlorophyll is the main feature in plant, especially in leaf areas that positively correlated with the photosynthesis rate. Chlorophyll synthesis is influenced by various factors such as light, sugar or carbohydrates, water, temperature, genetic factors, and nutrients such as N, Mg, Fe, Mn, Cu, Zn, S, and O (Hendriyani et al. 2009). The results showed that waterlogging and irradiation affected the chlorophyll of soybean leaves. The ANOVA test followed by the Duncan Multiple Range Test (DMRT) test showed that the irradiation and waterlogging treatment factors significantly affected the chlorophyll content.

Irradiation at 25 Gy is considered as the best dose compared to the others, while 100 Gy is the worst. The lowest chlorophyll content of 23.99 was found at 100 Gy with 250% waterlogging. Chlorophyll degradation can be shown through chlorosis in leaves under waterlogging stress (Pociecha et al. 2008). Chlorophyll decline occurred significantly in older leaves and this indicates the rapid degradation of chlorophyll in leaves near to the waterlogged roots. The decrease in chlorophyll content is suspected due to waterlogged damaged roots. Waterlogging in the soil causes plant roots to experience disturbances in respiration, absorption of nutrients and overall plant metabolism Kosova et al (2011). Waterlogging can cause the death of root cells which can inhibit the development and function of the root system such as the uptake of water and nutrients, especially nitrogen (N). As a result, the leaves wilt and turn yellow. Nitrogen plays a key role in the formation of chlorophyll in the leaves. The lack of nutrients causes the formation of chlorophyll to be disrupted and the chlorophyll content of the leaves decreases. However, in a long period of inundation conditions, inundation stress can cause a decrease in leaf area and the amount of chlorophyll, which in turn will reduce the amount of assimilation in plants (Sachs and Vartapetian 2007).

Number of pods

In addition to vegetative parameters, this study also measured one generative parameter, namely the number of pods. Measurement of the number of pods is one of the agronomic parameters to examine the effect of irradiation and waterlogging on soybean plants. The number of pods is related to the yield and makes it important parameters to be examined since it significantly reduced by waterlogging. The number of pods was counted at reproductive stage of set B soybean. After exposed to waterlogging stress at V2 stage, plants in set A were harvested, while plants in set B were treated normally with 100% of field capacity until R6 stage. Based on the results of the one-way ANOVA, it was found that irradiation dose factors did not affect the pods number ($p = 0.22$). On the other hand, waterlogging treatments significantly affected the pods number of plants ($p = 0.032$). However, it was observed that the increasing of irradiation dose resulting in the decreasing of plant productivity indicated by the less number of pod (Figure 1 G). Satpute and Fultambkar (2012) reported that induction mutations both physically and chemically can increase pollen sterility, causing an increase in the number of empty pods. Furthermore, waterlogging treatment worsen the plant productivity, in which at 100 Gy irradiation, the plants were only able to produce pods when they were in the control treatment and 100% waterlogging condition, while in the waterlogging conditions of 150% to 250%, the plants were unable to produce pods. This result was in linear with Ara et al. 2015 that found the reduction of pods number in four soybean genotypes under waterlogging treatment. Moreover, seven days of flooding at different vegetative and regenerative development stages significantly decline in pod number (Linkemer et al 1998). Ind addition, the number of pods per plant of *Vigna radiata* (L.) is the most sensitive parameter in responding to waterlogging stress compared to other yield components (Ahmed et al. 2002).

ISSR analysis of irradiated soybean plants in waterlogging condition

In this study, 10 ISSR primers were used to characterize the genomes of soybean plants that were able to survive in 250% waterlogging condition. The polymorphic information content (PIC) shows the informative level of primers, which means that the primers are able to attach to sites that experience an alteration due to mutations generated by irradiation process and amplify the fragment so that a polymorphism phenomenon occurs. The PIC value is divided into 3 classes, low (PIC value is less than 0.25), medium (PIC value is between 0.25-0.5), and high (PIC value is greater than 0.5) (Carsono et al. 2014). The details analysis of polymorphism generated by chosen primers is shown in Table 1.

The total number of loci detected from the ten ISSR primers was 68 with 16 polymorphism loci. The results showed that the irradiation caused changes in nitrogen base composition of the mutant lines detected by ISSR-1, ISSR-2, ISSR-3, ISSR-4, ISSR-6, ISSR-7, and ISSR-8 primers. The best primer in detecting variations in genetic material from irradiated soybean plants was ISSR-1 primer, which was in the medium category, resulting in the highest polymorphic bands with a PIC value of 0.381. Alteration in nitrogen bases is source of new variants in mutant lines. Regarding the results, the chosen primers showed a low rate of polymorphism, the utilization of more primers might be a proper way to further confirm the alteration. However, in this case, it is expected that the genetic changes occur only in genes associated or linked with the tolerance to waterlogging stress.

Protein profiles

Hypoxic conditions due to excess water in the rooting environment can trigger and increase anaerobic fermentation, which has an impact on morphological and physiological changes and various metabolic processes. These changes include photosynthesis, metabolic energy, redox potential, programmed cell death, and protein degradation or synthesis (Jackson and Ram, 2003). Plant has defense systems to encounter the stress, including changes in protein expression and post-translational protein modification (Hashiguchi et al. 2009). The results of protein profile analysis with SDS-PAGE in this study were varied, indicated by variations in thickness and degradation of protein bands in leaf organ as shown in Figure 3. The number of variations in protein bands showed the response of plants to waterlogging stress. Protein profile showed the change in hypoxic condition where specific proteins that were up-regulated.

Based on the results of SDS-PAGE analysis, there were several proteins with a molecular weight of 53.78 KDa which were overexpressed in soybean plants irradiated at 25 Gy with the waterlogging concentration of 250%. The protein was assumed to be 1-amino cyclopropane-1-carboxylate synthase (ACS) involved in the plants' response to waterlogging conditions. This is reinforced by studies that found protein with similar molecular weight in waterlogging stress in various plants as shown in Table 2. ACS generates the ethylene precursor by converting methionine to S-adenosyl methionine (SAM) and then SAM will be subsequently converted to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACS (Lee and Yoon, 2018). In the last step, ACC is finally converted to ethylene by ACC oxidases (ACO), a member of the oxygenase superfamily member (Bidonde et al. 1998). When low oxygen conditions exposed to the plants, it will induce the synthesis of ACS in root cap and cortex (Geisler-Lee et al. 2010; Argueso et al. 2007). In addition, a new protein appeared with a molecular weight of 43.12 Kda and was assumed to be an alcohol-dehydrogenase protein which was also widely involved in the plants response to hypoxia or waterlogging stress.

Table 1. Analysis of genetic variation generated by ISSR markers

Primers	Sequences	Tm (ºC)	Total locus	Total polymorphic locus	Percentage of polymorphic locus (%)	Total bands	Total polymorphic bands	EMR	PIC value	MI
ISSR-1	(AC)8G	53.0	9	4	44.00	45	12	1.776	0.381	0.6760
ISSR-2	(TCC)5GC	58.8	6	1	16.70	30	1	0.167	0.063	0.0105
ISSR-3	(GA)8T	45.4	8	1	12.50	40	2	0.125	0.095	0.0110
ISSR-4	(GT)8TC	51.6	4	1	25.00	20	2	0.250	0.180	0.0450
ISSR-5	(AG)8T	47.0	4	0	0.00	20	0	0.000	0.000	0.0000
ISSR-6	(CA)6GG	46.2	10	4	40.00	50	4	1.600	0.147	0.2352
ISSR-7	(CT)8GC	50.5	7	2	28.50	35	4	0.570	0.202	0.1150
ISSR-8	(GT)6CC	46.2	7	3	42.80	35	9	0.284	0.380	0.4870
ISSR-9	(CA)6AG	43.3	5	0	0.00	25	0	0.000	0.000	0.0000
ISSR-10	(CAC)3GC	44.7	8	0	0.00	40	0	0.000	0.000	0.0000
Total			68	16	209.5	340	34	4.772	1.376	1.5797
Average			6.8	1.6	20.95	34	3.4	0.477	0.138	0.158

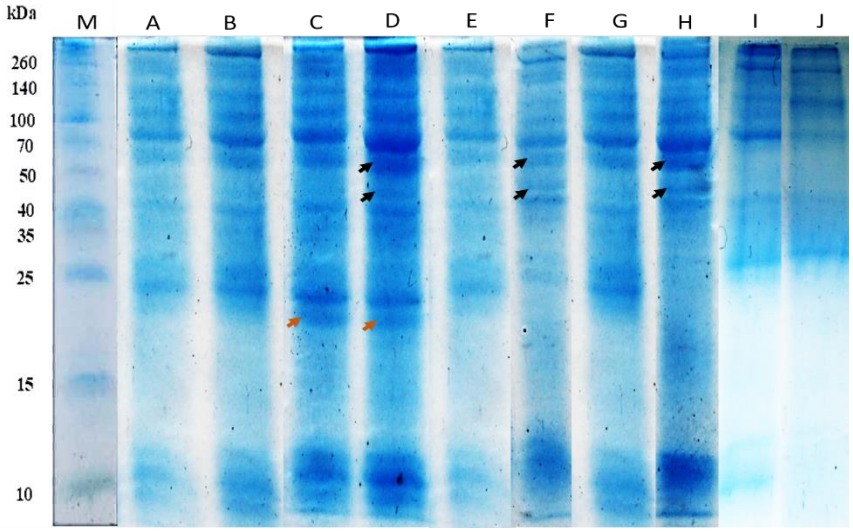

Figure 3. Protein profile of (A) Intial line in 0% of WS; (B) initial line in 250% WS; (C) 25Gy variants in 0% WS; (D) 25 Gy variants in 250% WS; (E) 50Gy variants in 0% WS; (F) 50Gy variants in 250% WS; (G) 75Gy variants 0% WS; (H) 75Gy variants 250% WS; (I) 100Gy variants 0% WS; (J) 100Gy variants 250% WS. Protein Ladder: spectra multicolor broad range protein ladder; WS = waterlogging stress.

Table 2. Protein prediction in waterlogging stress

Predicted protein	Organism	Accession	Amount of amino acids residues	Mol. weight (Kda)
1-amino cyclopropane-1-carboxylate synthase (ACS)	*Glycine max*	*(observed)*		53.78
	Glycine max	ABB70230	483	54.55
	Glycine soja	KHN40434	480	54.21
	Nicotiana tabacum	CAA67118	483	54.64
	Solanum lycopersicum	AAC32317	467	55.07
	Carica papaya	AAC98809	487	53.14
Alcohol-dehydrogenase (ADH)	*Glycine max*	*(observed)*		43.12
	Brassica napus	AGB57581	379	41.08
	Coix lacryma-jobi	ABE68381	379	40.97
	Actinidia deliciosa	ANA12162	380	41.33
	Barbarea vulgaris	AAF23556	379	41.16
	Glycine max (partial)	AAC97495	341	36.38
Superoxide dismutase (SOD)	*Glycine max*	*(observed)*		20.62
	Pisum sativum	BAC81657	152	15.10
	Arabidopsis thaliana	CAA43270	152	15.10
	Ipomoea batatas	ALP06096	152	15.08
	Brassica juncea	AAN60796	152	14.88
	Glycine soja	KHN38703	204	20.9

Expression of the alcohol dehydrogenase gene (ADH) in Arabidopsis is induced during hypoxia (Peng et al. 2001). ADH1 is essential for sugar metabolism via glycolysis to ethanol fermentation in both the embryo and endosperm (Takahashi et al. 2014). There were various proteins with sizes of 2-20 Kda widely involved in oxidative stress. Oxidative stress occurs when the production process and neutralization of Reactive Oxygen Species (ROS) in the cell do not run in balance. High light, heat, pathogen attack, low oxygen levels, and re-aeration after the hypoxic phase can increase ROS production (Suzuki et al. 2012). ROS is produced from molecular oxygen through several stages of reduction. Superoxide (O_2^-) anion, hydroxyl radical (•OH), and singlet oxygen are produced by reducing one or three electrons from oxygen with the reduction energy provided by the carrier electrons in mitochondria and chloroplasts (Chang et al. 2012). ROS is very reactive and can cause membrane and lipid damage (Meisrimler et al. 2014). Plants have a protective system to protect mitochondria from excessive ROS production such as antioxidants (glutathione, ascorbic acid, tocopherol, tannins, ubiquinol, and phenolic acid). ROS scavenging enzymes include superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione peroxidase (GPX). In this study, protein with size 20,62 KDa that predicted as Super Oxide Dismutase (SOD) also expressed in 25 Gy, 50 Gy and 75 Gy (Table 2). Furthermore, Seeds irradiated at 100 Gy either planted in control or waterlogging conditions had degraded proteins, causing them unseen on SDS PAGE. High irradiation caused damage to plant chromosomes, resulting in disruption of plant growth. Analysis protein using SDS-PAGE can only separate proteins based on their molecular weight. Furthermore, the protein sequencing needed to ensure that all protein predicted is truly 1-amino cyclopropane-1-carboxylate synthase (ACS), Alcohol-dehydrogenase (ADH), and Superoxide dismutase (SOD).

In conclusion, 25 Gy and 50 Gy gamma-ray irradiation consistently showed good performance in several growth parameters during waterlogging stress, while higher irradiation doses induced a negative effect. Irradiated lines

genetically differed to the wild type showing by the ISSR marker analysis amplifying 16 polymorphic loci. Those alterations were allegedly linked to their waterlogging tolerant status. Moreover, several proteins involved in plant defense during waterlogging stress were upregulated, which were suspected as 1-amino cyclopropane-1-carboxylate synthase (ACS), alcohol-dehydrogenase (ADH), and superoxide dismutase (SOD).

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