Nitrate reductase activity of black rice (oryza sativa l.) cempo ireng cultivar strain 13 and 46 as the result of plant breeding using 60Co gamma ray on drought stress variation

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Abstract. Black Rice (Oryza sativa L.) has been developed as a functional foodstuff due to its higher content of anthocyanins and nutrients. Black rice productivity is relatively low, while current drought stress phenomena may decline crop productivity. The breeding effort with gamma-ray irradiation is done to improve the plant's ability to be more tolerant against drought stress and increase productivity. Some researches mentioned the use of gamma-ray radiation could initiate morphological, physiological, and biochemical changes in plants. Nitrate reductase activity can be used as a method of crop yields estimation due to its positive correlation with crop productivity. The study aims to examine the nitrate reductase activity and growth responses of the M5 generation from Cempo Ireng Black Rice strain 13 and 46 results of 60Co gamma-ray irradiation compared to black rice without radiation on drought stress variation. The research was conducted with a completely random design factorial. Drought has become a factor I with 3 levels of spacious capacity (100%, 75%, and 50%). Factor II is the black rice strain (13, 46, and no radiation). The observed variables are growth parameters, nitrate reductase activity, and chlorophyll content. The data obtained were analyzed using ANOVA continued with Duncan test at a significance level of 5%. The results showed that there was a variation of growth, chlorophyll content, and nitrate reductase activities from black rice crops irradiated by gamma rays, which is also showed a different response to drought stress. The black rice strain of 46 is the rice, which more resistant to drought stress evaluated from the growth parameters, the content of chlorophyll, and its nitrate reductase activity is higher than black rice without radiation.

1. Introduction

Black rice is one of the sub-species of Oryza sativa L. in Indonesia, which has a characteristic of rice colored black due to the anthocyanin color pigment in the aleuron layer [6]. Anthocyanins are water-soluble pigments that are responsible for the antioxidative properties of black rice [6]. Anthocyanin has a protective effect against inflammation, atherosclerosis, carcinoma, and diabetes so that black rice has the potential to become a functional food ingredient [9].

The potential of black rice is not yet optimally utilized, one of the inhibiting factors is its low productivity, also long harvest time so that it has not been widely cultivated [7]. Efforts to meet the increasing demand for rice are also faced with various complex problems. The threat arises from global climate warming, which is one of the causes of drought, thus threatening agricultural productivity [8]. Black rice productivity needs to be increased as the compensation of long harvest time; besides that, it is necessary to develop varieties of plants that are more resistant to drought stress so that farmers can get maximum crops. One effort to overcome these weaknesses requires improvement in plant character to get superior rice through mutation induction techniques using gamma rays [10].

M5 generation black rice is a result of gamma irradiation with 60Co isotope; this mutation induction technique with gamma rays makes it possible to obtain superior strains [12]. Induction of mutations with
gamma rays can give rise to a different morphology with the parent plant, namely shorter stem and shorter harvest life. Black rice resulting from gamma-ray irradiation undergoes changes in physiological, biochemical, and nutrient content that affect its ability as functional food [10].

Conventional plant breeding can take a long time, so an effective selection method is developed to determine plants with high productivity. Nitrate Reductase Enzyme is the first enzyme that plays a role in reducing nitrates to nitrites, then becoming amino acids [17]. Nitrate Reductase activity (NRA) is related to plant productivity, so the level of Nitrate Reductase enzyme activity can be used as a selection criterion for selecting genotypes from high yielding plants [17]. This study aims to determine the growth response and nitrate reductase activity of irradiated Cempo Ireng black rice (Oryza sativa L.) strains of M13 and M46 under drought stress conditions.

2. Material and Methods

2.1. Research methods

This research was conducted for 4 months and was carried out at the Greenhouse of the UNS Central Laboratory, the UNS Biology Laboratory and the Laboratory of Integrated Mathematics and Natural Sciences Sebelas Maret University, Surakarta.

This research was conducted with a Factorial Complete Randomized Design (FCRD), consisting of two factors, namely drought stress as the first factor with three levels of treatment, each covering: K0 = 100% field capacity (KL) as a control, K1 = 75%, K2 = 50% and the second factor is the black rice strain (O. sativa L.), both gamma ray-irradiated like M13 (M1), M46 (M2) and non-irradiated black rice (control) (M0). Each treatment was carried out with 3 replications. The variables observed were plant height morphology, root and canopy ratios, measurement of plant biomass by calculating dry weight, chlorophyll content, and nitrate reductase enzyme activity.

2.1.1. Media preparation, measurement of field capacity, and seed quality testing.

The preparation stage includes the supply of planting media, 5 kg of soil sifted with a 2 mm x 2 mm sieve to get a uniform media structure. Planting media are made by mixing rice soil with compost with a ratio of 1:1. Measurement of the volume of water that must be splashed is done using the gravimetric method [4].

Watering is done every day in accordance with the level of watering that has been determined by each treatment. Seed quality testing includes germination and growth speed is carried out by groping rice on a tray with ±5 cm thick paddy soil media. Irradiation and Non-radiation seeds that are pithy are selected and then spread evenly. The seeds are watered periodically to maintain moisture. When the seeds are 3 weeks old, transplanting is carried out.

2.1.2. Planting Seeds

Seeds aged 3 weeks old are planted on soil media in prepared polybags. A polybag is filled with 3 seeds that are spaced apart. Watering up to field capacity is carried out every four days in all treatment units, adjusting to the time the soil media has reached perfectly drained conditions. When the plants are 14 days after planting (DAP), watering is done with different water volumes according to the treatment, which are 100% spacious capacity, 75%, and 50% spacious capacity.

2.1.3. Observation of research and testing parameters

Observations were made at 60 DAP after treatment, including growth, nitrate reductase activity, and leaf chlorophyll content. The growth parameters measured included plant height, dry weight and root-shoot ratio. Plant height is measured when the plant has been transferred to polybags, namely at 0 DAP, 15 DAP, 30 DAP, 45 DAP and 60 DAP. Measurements start from the ground level (certain signs) to the tip of the plant. Crops that have been harvested and will be calculated dry weight put in a paper bag then roasted at 60°C until dry, then weighed with an analytical balance. Plant roots and canopy are separated for measurement of root and shoot ratios as a guide for the presence or absence of drought stress.
Nitrate Reductase Activity was performed using leaf samples, the first leaf from the buds of 250 mg is sliced into small pieces, put in a dark tube containing 5 mL of phosphate buffer solution pH 7.2. After soaking for 24 hours the buffer solution is replaced and added with 0.1 mL NaNO₃ as a substrate, incubation is carried out for 2 hours. Coloring reagents consisting of 0.2 mL 0.02% N-Naphtylethylene diamine (NDN) were prepared in a test tube. After incubating for 2 hours, 0.1 mL of the filtrate is taken from a dark tube and put in a test tube containing a dye, then wait until a pink color appears as a sign of a reduction in nitrate to nitrite by the nitrate reductase enzyme. One test-tube was not given filtrate as a blank. Aquades 5 mL was added after the color change, transferred to the spectrophotometer cuvette, and absorbance was observed at a wavelength of 540 nm. Measurement of nitrate reductase activity is calculated by the formula:

\[
\text{Sample absorbance} \times \frac{100}{\text{BB}} \times \frac{1}{\text{WI}} \times \frac{50}{100} \, \mu\text{mol NO}_2^-/g/jam
\]

\( \text{BB} = \) sample wet weight (g)
\( \text{WI} = \) incubation time (hours) [2].

Chlorophyll content analysis was performed using leaf samples after harvested. The second or third black rice leaf from the top of 0.25 g was crushed using a mortar and added with 10 mL of 80% acetone. Each extract was then filtered and the filtrate was collected in a test tube. Absorbance measurements were carried out at \( \lambda \) 646 nm and 663 nm. Chlorophyll content values were measured using the formula (Moharana and Dutta, 2016).

- Chlorophyll a: 12.21 x (abs.663) - 2.81 (abs. 646) mg / L
- Chlorophyll b: 20.13 x (abs. 646) - 5.03 x (abs. 663) mg / L
- Total chlorophyll: 17.3 x (abs. 646) + 7.18 x (abs.663) mg / L

After measure the total chlorophyll content per liter of solution then the chlorophyll content per leaf weight is calculated with the formula:

\[
\text{Chlorophyll content (mg / g leaf weight)} = \left( \frac{10}{1000} \times \text{chlorophyll content ((mg / L))} \right) / 0.1.
\]

The results of measurements of plant height, plant biomass, shoot and canopy rasio, nitrate reductase enzyme activity and chlorophyll content were analyzed using One-way ANOVA to determine the effect of drought stress treatment (field capacity) with variations in seed strains. If it is significantly different, it is continued with the test followed by the DMRT test (Duncan Multiple Range Test) significance level of 5%.

3. Result and Discussion

3.1. Growth variables.
Growth variables in this study include plant height, plant dry weight, canopy and root ratio of plants shown:

![Figure 1](Image)

**Figure 1.** Average height of Cempo ireng black rice (O.sativa) strain M13 and M46 in variations of drought stress
Figure 2. Average dry weight of Cempo ireng black rice \((O.sativa)\) on drought stress variations

Figure 3. Average root and shoot/canopy ratio of \((O.sativa)\) Cempo ireng on drought stress variation

Figure 1 shows that increased drought stress gives the effect of decreasing plant height on each tested strain. The effect of drought stress began to be seen in observations 30 days after planting (DAP) to 60 DAP. The greatest decrease in height was seen in the M13 strain. M46 strain and control / non-irradiated plants showed an insignificant height decrease. It can be concluded that M46 strain and control / non-irradiated plants were not significantly affected by the presence of drought stress.

Drought stress decreases turgor pressure in cells, thereby suppressing cell expansion and growth. A decrease in shoot growth rate by drought stress is associated with inhibition of cell elongation. Under normal conditions, plant growth can take place optimally because water and carbon dioxide as raw material for photosynthesis will produce photosynthates that can be used for plants to grow [18]. The upland rice study by Supriyanto [18] reported that increasing stress intensity resulted in a decrease in plant height. Inhibition of cell enlargement that occurs due to decreased cell turgor results in smaller parts of the plant being formed [19].

Drought stress significantly decreases the dry weight of plants in each tested strain (Figure 2). The decrease in dry weight in the M13 strain was greater than in the M46 strain and in control / without radiation, with a decrease in the dry weight value from 7.9 g under conditions of 100% field capacity to 5.1 g at 50% field capacity. Dry weight is a manifestation of the accumulation of organic compounds that plants have successfully synthesized from inorganic compounds, especially water and CO\(_2\). Dry weight measurement is one of the parameters used to describe and study plant growth because it is easily...
measured and is an integration of all events experienced by plants, so that could explain the phenomenon of drought stress experienced by rice plants [12].

The average ratio of root-canopy of irradiated and non-irradiated Cempo ireng black rice (O.sativa) results can be seen in (Figure 3). Based on research conducted, drought stress does not significantly affect the root-shoot ratio for all strains, although there is a tendency for an increase in the root-shoot ratio. The absence of a difference in the root-shoot weight ratio indicates that the allocation of photosynthate is almost the same in both the shoot and the root. This is likely because plants are still in the vegetative phase, so not all organic material is allocated to parts of the plant exposed to stress but also depends on the photosynthetic source and photosynthetic sink.

The growth and root growth allometry is commonly expressed as the ratio of the root and shoot, which can describe one type of tolerance to drought. Root and crown homeostasis is the effort of plant organs to maintain physiological balance so that each organ can function normally [15].

An increase in the root-canopy weight ratio indicates that plants carry out a tolerance mechanism by increasing root development and growth compared to shoot growth and development. The root system is improved to be more efficient and effective in improving water absorption. This is consistent with the results of research on moth bean plants (Vigna aconitifolia) [16].

### 3.2. Chlorophyll content

![Figure 4. Average total chlorophyll content of leaves (mg / g) of Cempo ireng black rice (O.sativa) strain M13 and M46 in drought stress variations](image)

Based on research that has been done, the treatment of drought stress gives the effect of decreasing chlorophyll content in all black rice lines (O. sativa) Cempo ireng (Figure 4). Black rice without irradiation showed the most significant decrease in chlorophyll content compared to irradiated black rice. Irradiated black rice has decreased chlorophyll content but is not significant. The M46 strain shows a smaller decrease in value compared to the M13 strain.

The fact is strengthened in the morphological observation, black rice leaves turn yellow at the bottom due to the effect of drought stress. Decreasing the amount of leaf chlorophyll due to chloroplast damage due to the formation of H2O2 in the chloroplast stroma can lead to programmed cell death (apoptosis). Chloroplast organelle damage is triggered by the formation of ROS (Reactive Oxygene Species) [11].

In addition, the decrease in chlorophyll content due to drought stress is thought to be due to drought stress causing a decrease in plant rubisco enzymes. Rubisco is a representation of 25% leaf protein. Inhibition of photosynthesis causes a decrease in the supply of carbon to the cell, so that in the long run it will cause a decrease in cell synthesis [11].
3.3. Nitrate reductase activity

Figure 5. Average Nitrate Reductase Activity of Cempo ireng Black Rice (O.sativa) strain M13 and M46 in drought stress variations

Figure 5 shows that the highest nitrate reductase activity was recorded in the M13 strain with 100% KL. Increased drought stress was able to significantly reduce the NRA value of M13 strain, while M46 strain and control (without radiation) also decreased NRA but not significantly. The M46 strain showed the lowest decrease in NRA from 1,314 µmol NO2- / g / hour at 100% KL to 1,162 µmol NO2- / g / hour at 50% KL.

Increased drought stress will reduce NRA due to a decrease in the gradient of water potential between the environment and plant tissue, which plants respond to by closing the stomata mechanism on the leaves to maintain the plant's water potential. Closure of the stomata will cut the supply of carbon dioxide to mesophyll cells, as a result the rate of photosynthesis in these cells is reduced by the level of water shortage.

According to Fukuda et al. (2015), the smaller the efficiency of photosynthesis will affect the amount of reducing power (NADPH / NADH) produced so that electrons are not available in normal quantities. The resulting NADPH is not enough to support the activity of the nitrate reductase enzyme, so increasing drought stress can reduce nitrate reductase activity [17].

The results of research and measurements on each observation variable showed that the M46 strain had the smallest decrease in the value of the observed parameters compared to the M13 strain, and control plants / without radiation. The smallest decrease in value, especially in the parameters of dry weight, chlorophyll content and nitrate reductase activity show the ability of plants to defend themselves against drought stress.

Drought stress triggers a decrease in water potential. Low water potential in the environment creates a gradient of water potential between the environment and low or lost plant tissue. This condition makes it difficult for plants to take water from the environment into their tissues. The decrease in the potential of environmental water triggers the morphological response of stomatal closure to maintain the potential of tissue water, thereby inhibiting the process of transpiration [20].

The physiological aspect that is affected by the decrease in water potential due to drought stress is the chlorophyll content. The total amount of chlorophyll has decreased due to exposure to drought stress. Decreasing the amount of leaf chlorophyll occurs due to damage of chloroplasts caused by the formation of H2O2 in the chloroplast stroma that can lead to programmed cell death (apoptosis). Chloroplast organelle damage is triggered by the formation of ROS (Reactive Oxygen Species) [11].

Decreased chlorophyll content has an impact on decreasing the activity of nitrate reductase (NRA). The relationship of chlorophyll and NRA in this study was seen in nitrogen metabolism. Nitrogen (N) metabolism has a close relationship with photosynthesis. The light reaction of photosynthesis provides
energy to reduce nitrate to ammonium, one of which is reduced by the nitrate reductase enzyme. The process of plant photosynthesis is controlled by a pigment called chlorophyll. The function of chlorophyll in photosynthesis is as the capture of sunlight. The energy and reducing energy for nitrate reduction comes from cellular metabolism, namely carbohydrate respiration. Under normal conditions, the process of photosynthesis will increase the supply of carbohydrates and NADH needed for nitrate reduction resulting from carbohydrate respiration. Therefore chlorophyll levels have a close relationship with nitrate reductase activity.

Measurement of nitrate reductase activity enzyme (NRA) becomes a key character in plant selection, especially Cempo ireng black rice (O.sativa) results in predicting productivity because this enzyme is the first key in the pathway of synthesis of organic nitrogen compounds which have important aspects in the plant life cycle [17]. The results of research on a number of plants showed a positive correlation between NRA and yield. This correlation was found in the NRA growth and development phase of young plants with yield after entering the reproductive [17].

In this study, the mechanism of plant adaptation to drought stress in the form of modified root lengthening is characterized by an increase in root: shoot ratio. This mechanism is carried out by plants through increasing water absorption by increasing root depth and an efficient root system and reducing water loss through regulating the opening of the stomata and folding the leaves to reduce exposure to radiant heat, which can accelerate the rate of transpiration.

Referring to observable variables that have been measured, M46 strain has better resistance to drought stress conditions compared to M13 strain and control / no radiation. M13 strain is less resistant to drought stress but in conditions of 100%, field capacity shows the highest value, especially in the ANR value which is a predictor of crop productivity. The resistance of the M46 strain might be influenced by a number of physiological factors, presumably because plants develop a self-defense mechanism in the form of osmoregulation in the form of proline.

Proline is known to increase the binding capacity of water, so organelles do not dehydrate [1][14]. In addition, other studies state that the accumulation of proline as an osmoregulator also acts to protect and stabilize subcellular structures such as membranes and proteins from damage due to the formation of Reactive Oxygen Species (ROS) during stress conditions [21]. In addition, proline also plays a role in reducing radical oxygen derivatives in cells [5].

4. Conclusion
There are variations in the growth of black rice (O. sativa L.), nitrate reductase activity and chlorophyll content of Cempo ireng Black Rice under drought stress conditions. All growth variables include plant height, plant dry weight as well as root and shoot ratios, which showed that the M46 strain was more resistant to drought stress because the values between the parameters were more stable than the M13 strain and control / non-irradiated plants.

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