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Poliploidy induction of Indonesian Black Rice *Oryza sativa* L. var. Cempo Ireng with *Bio-catharanthine*

Ludfi Kurniawan[1], Alvina Nur Laili[1], Devi Silvia Anggaini[1], Salsabila Qurrotu 'Ain[1], Dyah Retno Wulandari[2], Fuad Bahrul Ulum*[1]

[1]Laboratory of Botany, Biology Department, Jember University, City Jember, Indonesia
[2]The National Research and Innovation Agency (BRIN)
* Correspondence Author: fuad.fmipa@unej.ac.id

Abstract

Black rice is a popular functional food due to its high antioxidant content and superior nutrition compared to white rice. However, its supply in the market remains limited due to low productivity and longer harvesting periods. *Oryza sativa* L. var. Cempo Ireng, a local black rice in Java, is widely consumed and is currently under intensive plant breeding development. Polyploid induction is one of the promising methods to improve plant characteristics. Anti-mitotic compounds such *e.g.* colchicine, catharanthine, and oryzalin have been applied in many studies for autopolyploid induction in plant breeding research. The aims of this study were to observe the effectiveness of *bio-catharanthine* for polyploidy induction in Cempo Ireng black rice and to analyze the induced black rice based on their stomatal cell, antioxidant activity, and chromosome features. This study used a two-factor treatment i.e. *bio-catharanthine* concentration (0.1 %, 0.2 %, 0.3 %, 0.4 %, 0.5 %, 1%, 1.5 %, 2 %, 2.5 %, and 3 %) and soaking durations (12, 24, and 48 hours). The results showed that *bio-catharanthine* did not reduce the germination rate of the Cempo Ireng seeds. The higher concentration (2.5 % and 3 %) and longest soaking duration (48 h) of *bio-catharanthine* altered the chromosome and increased the stomatal size and density of Cempo Ireng black rice. The antioxidants of the leaf sample did not alter by the treatment. This study proposed the application of *bio-catharanthine* with a promising potential for black rice polyploid research.

Keywords: antioxidant, *bio-catharanthine*, black rice, Cempo Ireng, polyploidy

Introduction

Rice is the staple food for 98 % of the population in Indonesia (Maligan et al., 2019). Pigmented rice has bioactive compounds in the pericarp grains that make the color brown, black, purple, and red. (Nabilah et al., 2022; Kristamtini et al., 2018). Some of these bioactive compounds are polyphenols, flavonoids, and antioxidants (Arifa et al., 2021). Black rice is currently getting popular as a functional food with health benefits (Kristamtini et al., 2018; Husna et al., 2022) in preventing cancer, free radicals scavenging, reducing blood cholesterol, and anti-inflammatory (Nurhidayah and Firmansyah, 2021).

Oryza sativa L. var. Cempo Ireng is one of the superior varieties of black rice that originated from Central Java and has been intensively studied for improvement through plant breeding projects (e.g. Pratiwi & Purwestri, 2017; Sutrisno et al. 2018; Fitri et al. 2021). The cultivation of Cempo Ireng was still rare in paddy fields due to several inferiority growth performances, i.e. required 150 days of harvesting ages with an average yield of only 4.5 tons per hectare compared to IR64 had a 120-day harvesting age and yielded around 6 tons per hectare (Nandariyah et al., 2020).

Improvement of plant characteristics through plant breeding strategy applied genome modification was suggested for food security and sustainable agriculture (Qaim, 2020). Polyploids in plants potentially form hybrid vigor with superior characteristics e.g. bigger cell size, higher biomass, and seed production, and resistance to biotic and abiotic stresses than their diploid progenitor (Ulum et al, 2021; Van de Peer et al. 2021). Polyploid induction is generally carried out using chemical antimitotic compounds as chromosome duplication agents, however, chemical antimitotic compounds are toxic to humans, especially when used in high concentrations (Wulansari et al., 2016). These toxic properties can be avoided by using natural antimitotic compounds such as *bio-catharanthine* from *Catharanthus roseus* extract which has the potential to be used as a polyploid induction (Amnah, 2021). This research aims to determine the effect of *bio-catharanthine* treatment on the germination rate of the seed, nuclear DNA content, antioxidant activity and stomatal size and density of the black rice Cempo Ireng.

Materials and Methods

Material

We used the seed of Cempo Ireng from e-commerce in Indonesia with a seed registered number 013798930. The *bio-catharanthine* was a commercial product of the Research Group of Biology Faculty of Gadjah Mada University.

Polyploidy induction

The research was carried out from June to October 2023 at the Botany and Biotechnology Laboratory, Department of Biology, Jember University. The treatment combination consists of 2 factors, i.e. concentration and soaking duration. The concentration of bio-catharanthine (0.1 %, 0.2%, 0.3 %, 0.4 %, 0.5 %, 1 %, 1.5 %, 2 %, 2.5 %, and 3 %) and soaking durations (12, 24, and 48 hours). The seeds were soaked for 4 hours for the seed selection and imbibition process (Nurrachmamila and Saputro, 2017). A total of 20 viable seeds were soaked in Eppendorf 2 ml tube with the *bio-catharanthine* solution. Seed germination was made on wet tissue paper at room temperature for 7 to 14 days (Anggraini et al., 2013). All treatments used three replications.

Germination rate

The number of normal germinated seeds is counted within 7 days. The characteristics of normal sprouts were a root system with primary and seminal roots, good hypocotyl development without tissue damage, perfect plumule growth with green leaves emerging from the coleoptile, and normal epicotyl growth with buds. The germination rate was calculated based on the percentage of germinated seeds among twenty seeds.

Flow cytometer analysis

Fresh paddy leaves were cut into 0.5 cm^2 and then placed on a petri dish. The leaves were dripped with 250 µl CyStain PI Absotule (nuclei extraction buffer) then chopped until fine using a razor blade and filtered using a 30 µm millipore sieve. The filtered filtrate was put into a cuvette tube and added with 350 µl of staining solution, propidium iodide, and RNAse. The sample was measured the DNA relative content with BD Accuri™ C6 Plus Flow cytometer for ploidy analysis (Hodač et al., 2016).

Stomatal size and density

The abaxial surface of a leaf was cleaned and smeared with transparent nail polish. After the nail polish dries, a strip of tape was attached to the area of the nail polish. The tape was removed slowly so the epidermis peeled off and then attached to the slide. Stomata characteristics were observed using a Nikon Eclipse E100 LED MV R microscope at 400x magnification connected to Optilab Advance by Miconos. Stomata length and width were measured using Image Raster software, while the stomatal density was calculated using the following formula (Lestari, 2006):
Stomata Density= (Number of Stomata)/(Area of Field of View (mm^2)

Antioxidant

Antioxidant activity was measured by the reduction activity of DPPH free radicals in the sample. Leaf samples were grounded with liquid nitrogen. Afterward, the samples were diluted with 70 % methanol and centrifugated at 4 ^{0}C for 15 minutes at 10.000 rpm. The supernatant was used for the measurement of DPPH radical scavenging. 100 µl extract was mixed with DPPH in a microplate and then incubated for 30 minutes. The absorbance of the samples was measured using a Bio-Rad microplate ELISA reader at 595 nm.

Statistical analysis

Data were analyzed with R version 4.1.2 for Windows (R Foundation for Statistical Computing). Statistical data visualization was using ggplot2 (Wickham, et. al. 2016). Significant differences were analyzed with the parametric (ANOVA) or non-parametric test (Kruskal Wallis). The post hoc with Student-t test, Wilcox test, or Duncan’s used Package ‘agricolae’ (de Mendiburu & de Mendiburu, 2019).

Results and Discussion

Germination rate

Bio-catharanthine had no toxic effect on the germination rate of Cempo Ireng black rice (Figure 1). The statistical analysis confirmed that in general germination rate of the seed was more than 80 % among treatments (Supplementary Table 1). This value was still as high as the control treatment. We had a single measurement of the germination rate from the control treatment was 90 %. The presence of lower germination with significant value might related to the variability of the seed viability. The production commercial seed of Cempo Ireng still does not exist. The current supply of seed originated from farmers or research institutions (Sutrisno et al. 2018). Albeit the highest concentration (3%) treatment revealed a high rate of germination rate among three different durations of seed induction with the *bio-catharanthine.* Several studies on the application of *bio-catharanthine* to polyploidy induction reported the viability performance of the subjected plant after the treatments with no toxicity symptoms (Rohmah et al, 2022; Shafura et al, 2022).

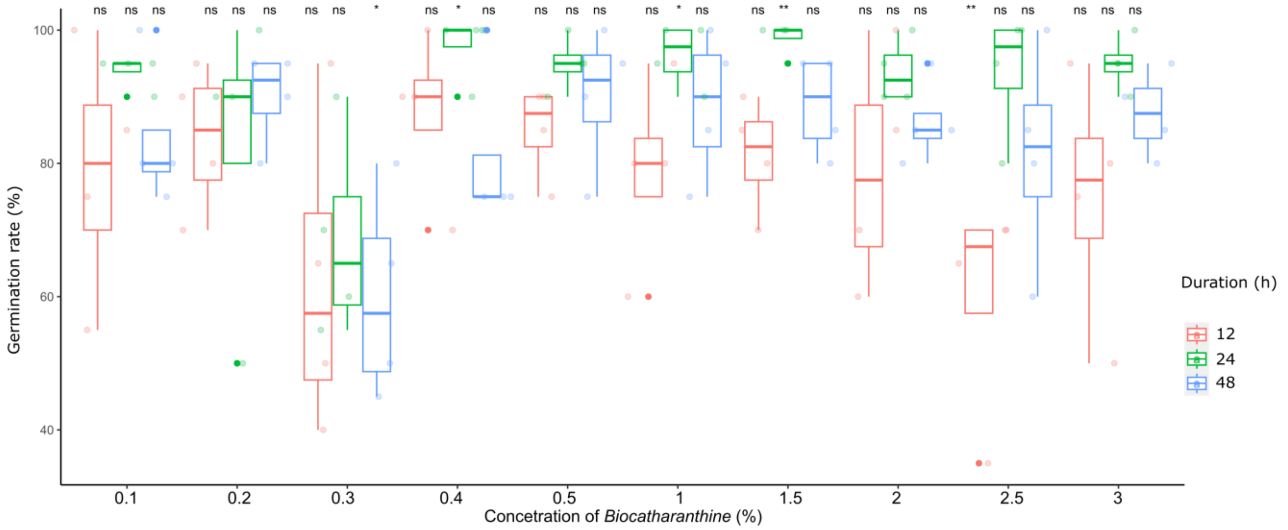

Figure 1. The germination rate of *Oryza sativa* L. var. Cempo Ireng seed under *Bio-catharanthine* treatment. Boxplot and whiskerplot represent the data distribution (25 and 75 percentile range and median). Jitter plots represent the exact data distribution. Summary statistic see Supplementary Table 1, * represent significant value ($p < 0.005$) of Wilcox test; ns = not significant

Ploidy Level

The diploid of Cempo Ireng black rice had DNA relative based on of Peak Index (PI) value of 53.39. Under the highest concentration treatment of 3 % *bio-catharanthine*, the ploidy levels of Cempo Ireng black rice were altered. The mutant of the Cempo Ireng black rice had a lower value of PI 38.07. The presence of the mutation in the set of chromosomes was also detected in the second-highest concentration of *bio-cahtaranthine* 2.5 % with a PI value of 51.5. On the other hand, the induction of *bio-cahtaranthine* in concentrations of 1 % to 2 % did not affect the ploidy level of the black rice (Figure 2). Research on polyploidy induction with a lower concentration of *bio-catharanthine* (0,1 – 0,4 %) reported two different effects of the compound. The chromosome number of *Allium cepa* L. var. ascalonicum 'tajuk' was altered into triploid and tetraploid (Billa et al, 2022), while no polyploids were observed from red spinach (Shafura et al, 2021). The lower value of PI value from the mutant shed light that the catharanthine compound influenced the mitotic division of the embryo cells of Cempo Ireng black rice. The inhibition of spindle formation by the catharanthine produces an unbalanced chromosome number of two daughter cells, resulting in a polyploid cell (Škubník et al. 2020). In this study, our sample had a lower chromosome as detected in 2.5 % and 3 % concentration treatment. With the presence of chromosome reduction in one daughter cell, there was also a high possibility of finding the other daughter cell with a higher chromosome number or polyploid. The putative tetraploid or higher polyploid paddy might be observed with the flow cytometry analysis through a higher number of population samples.

The duration of soaking treatment might be sufficient for the inhibition of spindle structure. A study on polyploidy induction with *bio-catharanthine* for 24 hours soaking treatment altered the *Arachis hypogaea* growth performance only at high concentrations (4 – 5 %) (Rohmah et al. 2021). All flow cytometer analyses were performed on the seed with a soaking treatment of 48 hours, which means two mitotic cycles which facilitate a higher possibility of the mitotic destruction. The longer duration of bio-catharanthine treatment in this study might still support the optimal condition for seed germination as presented in the germination data of this study.

Stomatal size and density

Induction of polyploidy with *bio-catharanthine* at high concentrations (2.5 – 3 %) increased the stomatal cell size of Cempo Ireng black rice (Figure 3 a-b). Wilcox test on the median values of stomatal length and width revealed that the alternation was significant from the treatment of 2.5 % and 3 % from all the soaking duration. The bigger stomata from the 3 % *bio-catharanthine* treatment were three times the size compared to the small stomata from the 1 % *bio-catharanthine* treatment (Figure 3 d-e). We presented only the concentration of *bio-catharanthine* from 1 to 3 % since the data from lower concentrations indicated they had smaller size (Supplementary Figure 1). The increase in stomata cell size was followed by the stomatal density. The stomata of Cempo Ireng induced by *bio-catharanthine* at a concentration of 3 % were still at the same density among all treatments. The student t-t test revealed that the density of stomata on the leaf of concentration 2.5 % and 3 % were not significantly different among the mean value of all data (Figure 3 c), whereas several leaves presented significantly lower stomata density from the treatment of 1 % 24 h, 1.5 % 49 h, and 2 % 12 h.

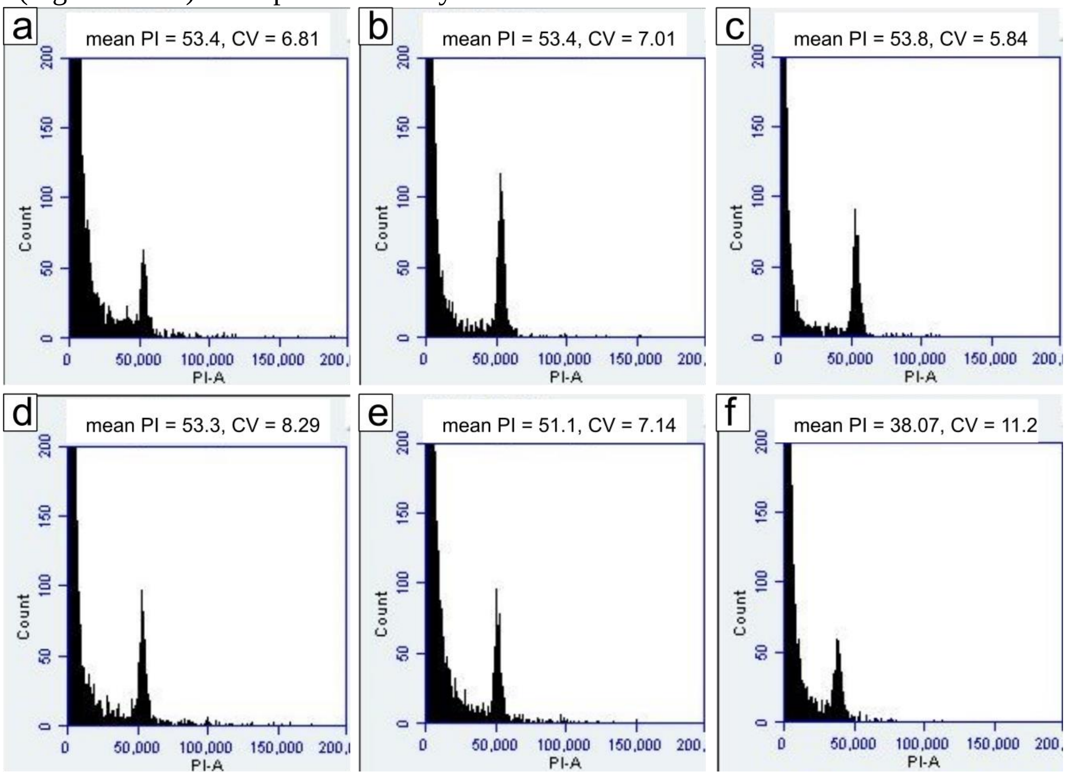

Figure 2. The ploidy level of leaf sample *Oryza sativa* L. var. Cempo Ireng under different concentrations of *Bio-catharanthine* treatment using a Flow cytometer. a) Control; b) 1 %; c) 1.5 %; d) 2 %; e) 2.5 %; f) 3 %; All sample were soaked for 48 Hour. Data presented DNA relative with mean value Peak Index (PI). Statistical data distribution is presented as CV value.

Polyploid plants with bigger cell sizes and biomass require a higher nutrient and photosynthesis performance (Ulum et al, 2021). Polyploid rice had bigger stomatal size and lower stomatal density compared to the diploid (Farhadi et al. 2023; Xiong et al., 2022). This study also reported the disadvantages of stomata enlargements in polyploid due to the speed of stomatal conductivity was faster in smaller size and denser stomata in diploid plants rather than the big and sparse stomata in polyploid rice (Xiong et al., 2022). In contrast recent study on polyploids by Šmarda (2023) reported that the polyploid had a better stomata conductivity to support the bigger size of stomatal cells and pores in relation to water and CO_2 efficiency. The increase of stomatal density in high concentration treatment of our study might related to the adaptation of the plant. Further research on the photosynthesis performance of polyploid Cempo Ireng under light stress conditions would be required to assess the heterosis of the plant.

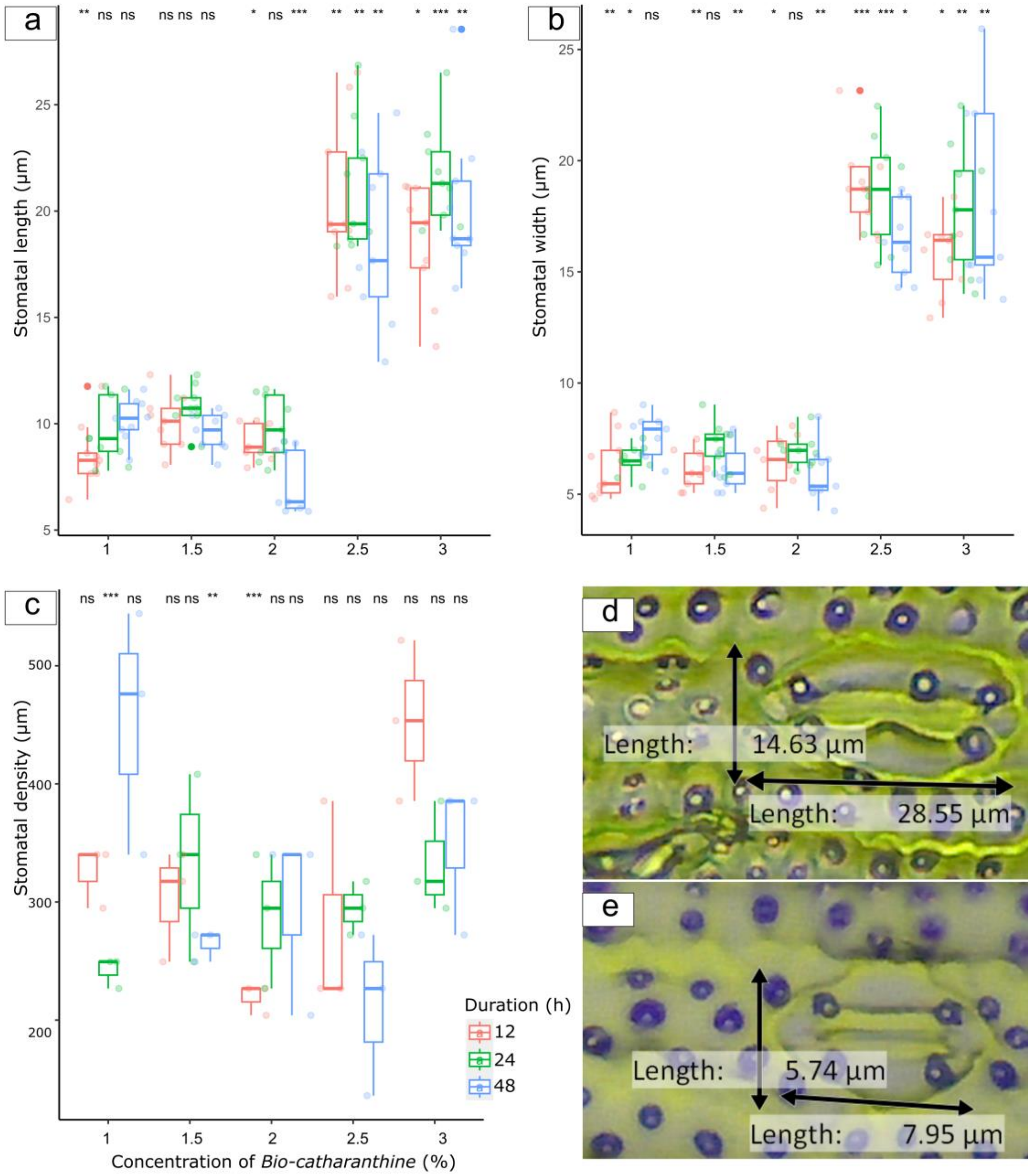

Figure 3. Stomatal size and density of *Oryza sativa* L. var. Cempo Ireng leaves under *bio-catharanthine* treatment. a) Stomatal length; b) stomala width; c) stomatal density; d) stomatal structure from 3 % of *bio-catharanthine* treatment; e) stomatal structure from 0.1 % of *bio-catharanthine* treatment. Boxplot and whisker plots represent the data distribution (25 and 75 percentile range and median). Jitter plots represent the exact data distribution. Summary statistic sees Supplementary Table 2, * represents the significant value (p < 0.005) of the Wilcox test or student t-test; ns = not significant.

Antioxidant

The polyploidy induction of Cempo Ireng seed with *bio-catharanthine* did not reduce the antioxidant activities of the leaves sample (Figure 4). The Duncan test revealed the significantly higher antioxidant activity of the leaf from control and treatments from higher concentration compared to the treatment of 1 % *bio-catharanthine*. The presence of heterosis from the data of stomatal size in our study was not followed by liner enhancement of antioxidants of the leaves samples. The study on the autopolyploid mutant of *Allium hirtifolium* reported that the polyploid mutant had a bigger morphology and produced greater chlorophyll and antioxidant activity (Farhadi et al, 2023). Nevertheless, the variation of metabolites alternation among polyploid were influenced by epigenetic factors (Schinkel et al. 2020; Xiang et al, 2023). In relation to this research, the high variation of antioxidant expression in Cempo Ireng black rice from 3 % concentration treatment might related to the epigenetic alternation among the polyploid mutants (Giraud et al., 2021). Here we confirmed that *bio-catharanthine* treatment in high concentration did not reduce the level of antioxidants of the Cempo Ireng black rice.

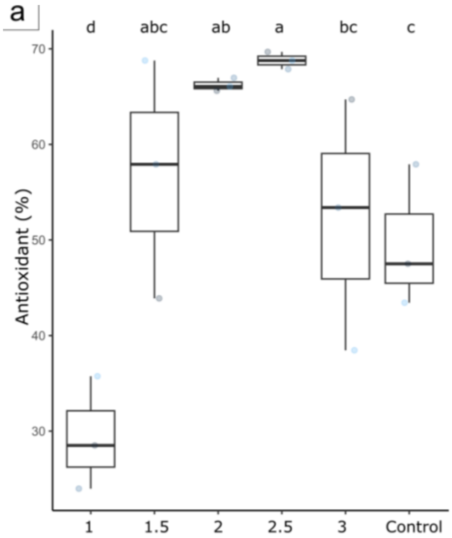

Figure 4. Antioxidant activity of *Oryza sativa* L. var. Cempo Ireng leaves under *Bio-catharanthine* treatment. Boxplot and whiskerplot represent the data distribution (25 and 75 percentile range and median). Jitter plots represent the exact data distribution. For summary statistic see Supplementary Table 2, Letter represent the significant value of the Annova-Duncan test

Conclusion

Polyploidy induction on *O. sativa* var. Cempo Ireng using *bio-catharanthine* with a high concentration (3 %) indicated the presence of chromosol mutation with an alternation of DNA content of the leaf sample. The antioxidant of the leaf Cempo Ireng black rice was not reduced by the treatment. The alternation of stomatal size and the non-toxicity effect of the compound on the plant made *bio-catharanthine* favorable for an alternative polyploidy induction.

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