Antioxidant activity of seedling growth in selected soybean genotypes (*Glycine max (L.) Merrill*) responses of submergence

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Abstract. In order to better understand the physiological and biochemical responses relating to direct seeding establishment in soybeans, the plant growth rate and antioxidative defense responses of seedlings in seven Indonesian soybean genotypes (Anjasmoro, Detam-1, Detam-2, Dieng, Grobogan, Tanggamus, and Willis) at different submergence periods (4, and 8 days) were examined. Twelve-day old seedlings were hydroponically grown in limited oxygen conditions. The results showed that the chlorophyll content in soybean seedlings was reduced beginning as early as 4 d under submerged condition, except for Detam-1, Detam-2, and Grobogan genotypes. The dry weight and protein concentration of seedlings were significantly higher at control condition (0 d) than those in submerged condition. The activities of superoxide dismutase (SOD) increased linearly until 8 d submerged for all genotypes. On the other hand, our results showed that catalase (CAT) and ascorbate peroxidase (APX) activities did not work together, meaning that CAT is activated and APX deactivated, or vice versa, in response to submergence conditions, except for Grobogan and Tanggamus genotypes which had an effect on both CAT and APX activities. Submergence stress led to a significant increase in glutathione reductase (GR) together with APX activity for Detam-2 and Dieng genotypes at 8 d submerged.

1. Introduction

The soybean is one of the most important food commodities in Indonesia after rice and corn. About 60% of the national soybean production comes from the marginal lands and rice fields, therefore the harvest has fluctuated. Soybean planting at rice fields comes with many problems, especially flooding, which causes premature senescence that results in leaf chlorosis, necrosis, defoliation, cessation of growth, and reduction of yield.

In higher plants, the adverse effects of abiotic restrictions (submergence, salinity, drought, heat/cold, light) are dependable under oxidative stress which happens by an antioxidant defense system in support of the plants [1-2]. In fact, a common response to the stress causing all these adverse conditions is an oxidative burst, which means there is a temporary overproduction of the reactive oxygen species that results in cell death, tissue damage, and reduced yield.
oxygen species (ROS) within the diverse cellular stall of the plant cell [3]. ROS have divided into two main classes consisting of non-radical species ($\text{H}_2\text{O}_2$) or free radical forms $\text{O}^-$; $\text{OH}^-$; $\text{OH}^2_-$. Accumulation of high concentrations of ROS is potentially detrimental to plants cells causing damage to valuable biomolecules like DNA, proteins, lipids, chlorophyll, and membranes [4].

Plants are strictly affected by environmental tension because under such stress the production and quenching of the reactive oxygen species (ROS) in plants will stop working in order to maintain the impartial state. One of the major biological consequences of soil flooding is oxygen deficiency. Roots suffer from periodic or prolonged deprivation of oxygen, which interferes with respiration at the level of electron transport. The lack of a suitable electron acceptor leads to saturated redox chains, accumulation of NAD (P)H, and a decline in the generation of ATP. The toxic radicals can be removed through the mobilization of antioxidant reserves, which react both enzymatically and chemically with the toxic molecular species and their products. Compound constituents have been recognized to scavenge free radicals and consequently defend active plant cells adjacent to oxygen toxicity. To counteract the hazardous properties of ROS while under stress, plants have evolved a multifarious antioxidative defense system composed of antioxidant enzymes and metabolites such as ascorbate peroxidase (APX), catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR), ascorbic acid (AsA), glutathione (GSH), oxidized glutathione (GSSG), and Vitamin E [4-5].

The construction and scavenging of activated oxygen species are correlative, which maintains the plants in a reasonably steady state. Plants are modified to minimize radical damage using their strategy protection mechanisms. Thus, the sense of balance between the arrangement and detoxification of activated oxygen species is significant to cell endurance during flooding stress. [4].

2. Materials and methods

1.1. Plant materials and stress condition

The study was conducted using seven soybean genotypes to examine the differences in their tolerance of submergence at the early seedling stage. All those genotypes were provided by Indonesian Agricultural Research Centre, Malang, Indonesia. The experiments were conducted in laboratory conditions with temperature maintained at 25 ± 2°C. The healthy seeds were surface sterilized in an aqueous elucidation of 25 mM sodium hypochlorite for 25 min, rinsed four times with distilled water, and germinated on two sheets of Whatman filter paper (No 1; Macherey-Nagel, Germany) in 300 ml bottles moistened with 5 ml distilled water in dark conditions for 4 days. Three replicates of 20 seeds in each bottle were used for germination. The germinated seeds were maintained and continued to grow in the bottles and watered for 1 week before submergence treatment. It was designed for the experiment that 12-day-old seedlings were completely submerged in tap water for 4, 8, and 12 d in a concrete glass tank (dimension 50 cm x 20 cm x 20 cm) under 12 h light period/day and 70% relative humidity, with water levels maintained at 10 cm above the seedlings. Non-stressed seedlings were cultured in a similar concrete glass tank, but without the filled with water. A sufficient number of bottles were used within each replicate to provide adequate plant material for daily sampling during the first 4 d following the beginning of the experiment. During the submerged period, germinated seeds were kept submerged until measurements were completed.

1.2. Determination of plant biomass and chlorophyll analysis

Plants were harvested prior to stress imposition 4, 8, and 12 d after the start of submergence treatment. For each submergence duration, 10 plants (shoot + root) were completely submerged in tap water for 4, 8, and 12 d in a concrete glass tank (dimension 50 cm x 20 cm x 20 cm) under 12 h light period/day and 70% relative humidity, with water levels maintained at 10 cm above the seedlings. Non-stressed seedlings were cultured in a similar concrete glass tank, but without the filled with water. A sufficient number of bottles were used within each replicate to provide adequate plant material for daily sampling during the first 4 d following the beginning of the experiment. During the submerged period, germinated seeds were kept submerged until measurements were completed.

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centrifuged at 5000 g for 10 minutes, and supernatants were measured at absorbance $A_{645}$ and $A_{663}$. Calculation data used the equation: total chlorophyll ($\mu$g/ml) = 20.2 ($A_{645}$) + 8.02 ($A_{663}$).

1.3. Preparation of crude enzyme extracts
Three repititions of clean plants were collected from the bottles. The collected plants were treated with liquid nitrogen in a mortar and were homogenized with ice frozen 50 mmol L$^{-1}$ sodium phosphate buffer (pH 7.8) containing 0.1 mM ethylenediaminetetraacetic acid (EDTA).Na$_2$ and 2% (w/v) insoluble polyvinyl polypyrrolidone (PVPP). Then, the homogenate was centrifuged in an eppendorf tube for 25 min at 10 000 g and the resultant supernatant was used for enzymes activity measurements. The soluble protein content of the soybean seedlings was determined by the method of Bradford [7].

1.4. ROS scavenging enzyme assays
The assay for superoxide dismutase (SOD, EC 1.15.1.1) activity was estimated according to the method of Steward and Bewley (1980) using nitroblue-tetrazolium (NBT) [8]. The reaction mixture contained enzyme, 390 mM L-Methionine, 2.25 mM NBT, 1 mM EDTA, 7% Na$_2$CO$_3$, 0.05 mol L$^{-1}$ Na-Phosphate buffer (pH 7.8), and 60 µM riboflavin. The riboflavin was added last to start the reaction. The reaction mixtures were placed 30 cm from the light source (about 15W fluorescent lamps) for 10 min, and the absorbance decrease was recorded at 560 nm. The non-irradiated reaction mixture without enzymes, which gave the maximal color, served as the control and was deducted from $A_{560}$. One unit of SOD activity was defined as the amount of enzyme that caused 50% inhibition of the initial rate of reaction in the absence of enzyme, expressed in units min$^{-1}$ mg protein$^{-1}$.

Catalase (CAT, EC 1.11.1.6) activity was assayed by measuring the initial rate of disappearance of H$_2$O$_2$ [9]. The extraction mixture contained 300 µL of enzyme extract, 0.5 mL of 10 mM H$_2$O$_2$, and 600 µL of 30 mM potassium phosphate buffer (pH 7.0), and the decrease in H$_2$O$_2$ following the decline in absorbance was recorded at 240 nm for 30 s. The enzyme activity was calculated as µmol H$_2$O$_2$ oxidized mg$^{-1}$ protein min$^{-1}$ by using an extinction coefficient (€ = 36 µM$^{-1}$ cm$^{-1}$).

Ascorbate peroxidase (APX, EC 1.11.1.11) activity was measured following the method of Nakano and Asada [10]. The assay mixture contained 100 µL enzyme extract, 600 µL 0.1 mM EDTA in 0.05 M Na-phosphate (pH 7.0), and 400 µL 0.5 mM Ascorbic acid. The reaction started with the addition of 400 µL of 3% H$_2$O$_2$ and the absorbance decrease was recorded after 1 minute at 290 nm. The activity of APX was calculated using an extinction coefficient (€ = 2.8 mM$^{-1}$ cm$^{-1}$).

Glutathione reductase (GR, EC 1.6.4.2) activity was estimated following Goldberg and Spooner [11]. The assay mixture contained 100 µL of enzyme, 2.5 ml of 0.1 mM EDTA in 0.05 M Na-phosphate (pH 7.0), and 100 µL 0.5 mM oxidized glutathione (GSSG). After 5 minutes 50 µL of 9.6 mM NADH was added and mixed thoroughly. The absorbance decrease was recorded at 290 nm spectrophotometrically at an interval of 1 minute. The expression of 1 unit of GR activity is nmol glutathione reduced per minute, calculated using an extinction coefficient (€ = 6.22 mM$^{-1}$ cm$^{-1}$).

1.5. Statistical analysis
All the data obtained was subjected to a one-way analysis of variance (ANOVA), and the mean differences were compared by a lowest standard deviations (LSD) test. Comparisons with $P < 0.05$ were considered statistically significant.

2. Results
2.1. Plant biomass
The plant biomass of the seedlings was observed, and the dry weight of plants was determined as shown in Figure 1. Although the seven soybean genotypes were able to grow under stress conditions, we observed a significant difference in the biomass response of the seedlings.

The biomass of the genotypes significantly decreased during submergence periods both for the tolerant (Dieng) and intolerant genotypes compared with their controls, with a large decline in the
intolerant genotypes. The longest period of submergence 8 d led to decreased biomass of the seedlings. The accumulation of biomass for intolerant genotypes ranged from 42% to 47%, whereas the accumulation of biomass in the tolerant genotype Dieng was 54% and almost the same as intolerant genotypes Detam-1 (56%) and Detam-2 (57%) compared to their respective control plants. Although the intolerant genotypes of soybean seedlings seemed to have higher contents of dry weight compared with the tolerant cultivar, submergence stress caused differential reductions that were greater in intolerant genotypes than in the tolerant Dieng genotype. The lowest depletion of biomass at 8 d submergence was at the Willis genotype, compared with the control.

Figure 1. Changes in dry weight of soybean seedlings genotype at different days of submergence.

2.2. Effect of submergence on chlorophyll analysis
To investigate the physiological responses to submergence during the growth of the early seedlings of soybeans, the total chlorophyll substance was determined. The total chlorophyll content showed diverse profiles depending on the genotypes (Figure 2). Relative to the control, a marked decrease in chlorophyll was observed in submerged plants at 4 d except for Detam-1 and Grobogan genotypes, which maintained higher total chlorophyll content compared to the control. Accumulation of chlorophyll progressively declined when extending the period of submergence treatment, and remained significantly lower compared to the non-submerged seedlings in the tolerant genotype.

Figure 2. Changes of chlorophyll content of soybean seedlings genotype at different days of submergence.
2.3. Effect of submergence on antioxidant enzymes activities

A considerable discrepancy change was detected in the actions of the antioxidant enzymes within responses to submergence treatment in all genotypes. Under submerged conditions, the superoxide dismutase activity in all genotypes was higher than the control. The activity of SOD was greatly increased at 8 d for all genotypes except for the Willis genotype (Figure 3). For the soybean-tolerant Dieng genotype, SOD activity under 4 d and 8 d submerged were 14 times and 5.7 times higher than the control, respectively. Detam-1 and Detam-2 genotypes recorded a continuous increase in SOD activity up until 8 d submerged higher than the control, respectively. The establishment of superoxide dismutase through submergence stress is probably related to increased production of hydrogen peroxide, which is scavenged by peroxidase and/or catalase. Peroxidase and catalase activity was considered for soybean plants in different submergence periods.

![Figure 3. Superoxide dismutase activity of soybean seedlings genotype at different days of submergence.](image)

The response of CAT action to submerged stress varied with the genotype and submerged periods. There was a considerable increase in catalase activity detected for Detam-1 and Detam-2 genotypes, but not for the flood-tolerant Dieng soybean genotype compared to control at 4 d submerged (Figure 4).

![Figure 4. Catalase activity of soybean seedlings genotype at different days of submergence.](image)
The ascorbate peroxidase (APX) activity of the Anjasmoro, Grobogan, and Willis genotypes showed a significant decreased under 4 d submerged seedlings, although APX activity still decreased until 8 d submergence compared with the control seedlings (Figure 5). The consequences also showed that APX activity significantly increased for Detam-1, Detam-2 and Dieng genotypes compared with the control seedlings starting at 4 d and 8 d submerged.

![Figure 5: Ascorbate peroxidase activity of soybean seedlings genotype at different days of submergence.](image)

The glutathione reductase action in submerged seedlings showed a considerable difference between submergence treatments, for all genotypes. The Detam-2 genotype had increased GR activity after 8 d submerged by almost twenty-folds, relative to the non-submerged control (Figure 6).

![Figure 6: Glutathione reductase activity of soybean seedlings genotypes at different days of submergence.](image)

3. Discussion
The capability to continue to exist under particularly elevated water for flood-tolerant soybean genotypes such as Dieng (Figure 1) was related to the sufficient biomass and to quick regeneration growth (shoots and roots) before and after the water stage decreased [12]. Moreover, the genotype anticipated the properties of shoot elongation competing with maintenance processes for energy. For that reason, energy running appears to be crucial for submergence acceptance. For example, the
quantity of starch and total carbohydrates before and after submergence seemed to have a considerable impact on favored survival compared with the regeneration of the plant [13].

In this study, we have investigated the characteristics of soybean plants treated under non-submergence conditions, which appeared progressively green, and the level of chloroplast pigment was as expected. The seedlings began to display evident symptoms during a 4-day period of submergence, such as some leaves developing chlorosis and turning yellow, which was followed by defoliation at the bottom of the stem segments. Some of the genotypes maintained their membrane integrity during 4 d submerged (Figure 2), which then decreased during longer submergence periods. This was possibly due to the fact that competence for submerged photosynthesis was decreased between intolerant genotypes, which depend on energy consumption to survive prolonged submergence. Carbohydrate starvation has been shown to be one of the possible reasons for hypoxia/anoxia-induced injuries [14].

Aerobic respiration was inhibited due to oxygen deficiency, and consequently, the carbohydrate and energy status dropped to harmful levels in susceptible submerged plants [15]. Also, under submergence conditions, the hasty drop in the CO\textsubscript{2} photosynthetic assimilation rate was probably due to severely limited CO\textsubscript{2} because of the small absolute concentrations involved compared with O\textsubscript{2} and the structural damage suffered by the photosynthetic apparatus as evident from the fall in the values of Fo, Fm and Fv/Fm ratio. In addition, an irreversible loss in Rubisco activity and plant pigment content might be responsible for a poor CO\textsubscript{2} photosynthetic assimilation rate [12,16].

There is no doubt that submersion can cause anaerobiosis and make plants suffer from anoxia. The enhancement of the production of antioxidant enzymes may play an important role in metabolic stress tolerance. Plants have evolved an enzymatic mechanisms to scavenge the rapidly evolving ROS under stress. The effects of submergence stress on soybean plant genotypes were monitored by tracking changes in antioxidative enzyme activities, SOD, CAT, APX and GR which work in concert to detoxify ROS on the various submergence days of treatment. The changes in CAT may vary according to the intensity and time of stress. The activity of the enzyme ascorbate peroxidase showed a significant reduction in the Willis and Anjasmoro genotypes compared to control plants (Figure 5). The level of antioxidative reaction depends on the species, the development and the metabolic state of the plant, as well as the duration and intensity of the stress [17]. CAT scavenges H\textsubscript{2}O\textsubscript{2} to water, but it has a lower affinity for H\textsubscript{2}O\textsubscript{2} than APX [18]. The correlation action between SOD and CAT activity under a pressure of submersion showed a match for Anjasmoro and Willis genotypes (Figs. 3, 4), while the combination of SOD and APX was found in Dieng, Grobogan and Tanggamus genotypes (Figs. 3, 5). CAT is known as an enzyme that works to eliminate the negative effects of hydrogen peroxide, and aligns with APX to accomplish the same tasks as neutralizing hydrogen peroxide [17].

In our experiments, we found a gradual increase of GR among the submerged seedlings in the 8 days of submergence for all genotypes compared with the control. The improvement of GR enzyme activity also plays an important role in abiotic stress. Glutathione is a crucial antioxidant associated with regenerating AsA in the AsA-GSH cycle, and thus GSH is also involved in the regulation of H\textsubscript{2}O\textsubscript{2} concentrations and control of the redox state in plant cells [19]. The relationship between GR functions and APX activity under stress conditions (in the AsA-GSH cycle) has been well recognized [4, 15, 19]. Hence, the increase in GR activity alone is not sufficient to put off stress induced H\textsubscript{2}O\textsubscript{2} production. In the present study, APX activity increased at 8 d submerged for Detam-2 and Dieng genotypes, which correlated with the improved in GR activity at the same days of submerged (Figs. 5, 6).

Attention to the Detam-2 genotype can be used for tolerance to flooding, in addition to the Dieng genotype, which is known to be tolerant to flooding. This also shows that the activity of enzyme GR can be used as a criteria to evaluate tolerance flooding soybean genotypes. However, the evaluation of biochemical aspects needs to be further explored in order to analyze the enzyme activity of GR in cooperation with other antioxidants associated with cycles of AsA-GSH in the regulation of stress signals on soybean plants.
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