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Different Nitrate and Ammonium Levels Media on Changes of Nitrogen Assimilation Enzymes in Rice

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Abstract

Nitrogen (N) is an important nutrient for the growth and development of rice plants, required in large quantity and often limiting factor of rice yields. The research was to understand the different sources and levels of nitrogen in rice plant on the activity of N assimilation enzymes, including content of nitrate reductase (NR), glutamine synthase (GS) content, glutamate synthase (Gogat) content, content, ammonium (NH_4^+) and nitrate (NO_3^-) content on the leaves. Paddy (Ciherang variety) was grown in sand media containing Hoagland solution with different sources (ammonium and nitrate) and levels (0.4, 0.8, 1.6, 3.2, 6.4, and 12.8 mM) of nitrogen. Nitrogen assimilation was observed from leaves at one month of age. The NR activity increased on both Nitrogen sources, it was a higher activity in media contained nitrate. Also, the activity of GS showed higher in media contains nitrate, but its activity was decreased after application 1.6 mM of nitrate and 3.2 mM of ammonium. Western blot analysis of GS1 and GS2 showed that the band pattern of protein was similar to these enzyme activities. Nitrate content in leaves gradually increased in both sources of nitrogen and higher than 3.2 mM ammonium application caused an increase in ammonium content in leaves, but the nitrate content decreased. This research resulted that the available source of N for rice was in nitrate form, easily uptake by the rice plants during the growth stage.

Keywords : nitrate reductase, glutamine synthase, glutamate synthase, nitrate, ammonium

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1. Introduction

Nitrogen is essential macro nutrient for the growth, development, and reproduction of plants, required in the growth of vegetative parts such as stems, leaves and roots, which also play a key role in nitrogen assimilation. Nitrogen promotes the growth and development of plants quickly. However, excessive nitrogen can degrade the quality of the plants [1]. Generally, rice plant absorbs very low nitrogen, approximately 30% by broadcasting in the irrigated field [2]. It absorbs nitrogen from the soil in the form of ammonium (NH_4^+) and nitrate (NO_3^-) and required in the form of NH_4^+ more than in the form of NO_3^-, this is because NH_4^+ ion in a reductive state will be unstable so that ammonium is accumulated in the root zone of paddy [3]. Ammonium availability is beneficial for the growth of rice plant.

Nitrogen assimilation is the formation of organic nitrogen compounds from the nitrate and ammonium form converted to amino acids. Several enzymes play the important role in nitrogen assimilation i.e. nitrate reductase (NR), glutamine synthetase (GS), and glutamine-2-oxoglutarate aminotransferase (GOGAT). NR catalyzes NAD(P)H reduction of nitrate to nitrite [4]. Its activity increased when the nitrate concentration increased. Glutamine synthetase and Glutamate synthase catalyze glutamate conversion from the α-ketoglutarate and ammonia. Some researchers reported that the GS activity was also influenced by the concentration of ammonium. GOGAT catalyzes the reduction of the glutamine amide group to form two glutamate molecules [5]. NR as an enzyme that plays a role in the assimilation of nitrogen had the highest activity in young leaves and was fully developed [6]. The increasing age of the leaves will cause a decrease in the activity of NR.

GS is an enzyme that catalyzes ammonium into glutamine, using ATP as energy and glutamate as substrates. It has a very important role in controlling the process of nitrogen metabolism. GS together GOGAT catalyzes the transfer of the amide group of glutamine into α-ketoglutarate to form two molecules of glutamate. GS has a high affinity for ammonium and works in a low ammonium concentration at the Km 3 to 5. In plants, there are two types of GS i.e. type I is in the cytosol, and GS type II which is in the chloroplast [7]. Both forms of this enzyme found in the roots of soybean and peanut. Holoenzyme-shaped GS has a molecular weight of 38 kD and 41 kD [8]. Physiological studies on the absorption of N are very important to learn because it involves many enzymes. Physiological and biochemical research on rice plant has been of great interest, especially concerning the absorption of nitrogen in the form of nitrate or ammonium toward the nitrogen assimilation enzymes including NR, GS, and GOGAT.

2. Materials and Methods

2.1 Plant material preparation

Ciherang variety was obtained from the seed store and was used extensively for the study of enzymes. Seeds were soaked in fungicide for 24 hours, then in water for 3 days, followed by germinating in sand media for 6 to 9 days. Germination was kept in a growth chamber (Programmable Growth Chamber WGC-450, Daihan Scientific. Co. Ltd.) at day/night (12/12 hours) temperature of 30ºC. Uniform seedlings were transplanted to black polyethylene pots and grown hydroponically with the different ammonium and nitrate concentration (0.4; 0.8; 1.6; 3.2; 6.4; 12.8 mM) of Hougland formulation in the sand media. Nutrient solutions (500 mL) were supplied once a day and additional water was supplied if required.

2.2 Extraction and measurement of the activity of enzymes

Protein extraction. Two grams of fresh weight leaf was homogenized with 6 mL extraction buffer (100 mM Tris-HCl pH 7.5, 0.5 mM EDTA, 5 mM $MgCl_2$, 10% of insoluble polyvinylpyrrolidone, 1 mM dithiothreitol) in 50 mL centrifuge tubes using a Turrax homogenizer for 2 minutes. Extracts were squeezed through cheese-cloth, centrifuged at 4°C at 50,000 g for 10 min and 2 mL of the supernatant were desalted at 4°C on the Sephadex G25 gel filtration column (20x150 mm) that had been equilibrated with a buffer containing 50 mM Tris-HCl pH 7.5, 25 mM EDTA, 2.5 mM $MgCl_2$, 0.5 mM dithiothreitol. Enzymes assays were carried out immediately.

Glutamine synthetase activity. A 0.5 mL of supernatant was added in 0.5 ml assay mixture solution containing 0.34 μM adenosine diphosphate (ADP), 5 μM manganese chloride ($MnCl_2$), 34 μM hydrochloride, 130 μM L-glutamate, 66 μM sodium arsenate, and 200 μM Tris-acetate (pH 6.4) and 0.5 ml of distilled water. The mixture was incubated for 0, 15, and 30 min at 30°C and then stopped the activity of the enzyme by adding 0.5 ml of a solution containing 0.74 mM ferric chloride ($FeCl_2$), 2 N hydrochloric acid (HCl), and 0.4 M trichloroacetic acid (TCA). The precipitated form was centrifuged and was checked using a spectrophotometer at a wavelength of 500 nm. GS activity was compared with standard glutamyl hydroxamate.

Nitrate Reductase activity. 0.5 ml of supernatant was added to 1 ml of solution containing 25 mM K-phosphate (pH 7.5), 10 mM potassium nitrate (KNO_3), and 0.2 mM nicotinamide adenine dinucleotide (NADH) and add distilled water to a volume of 2 ml. After that, the mixture was incubated at 30°C for 0, 15, and 30 minutes. The enzyme activity was stopped by adding 1 ml 1% Sulfanilamide in 1.5 N hydrochloric acid and of 0.02% N-(1-naphtyl) ethylenediamine dihydrochloride. The mixture was shacked and incubated for 30 minutes. Nitrite was checked by a spectrophotometer at 540 nm. NR activity compared with a standard nitrite 0-20 nM.

Ammonium and nitrate content. Ammonium content was measured according to the modification method of [9]. Two grams of leaves was extracted 3 times by 6 mL of 80% ethanol. Then the ethanol was pooled and evaporated until the volume was equal to the weight of the leaves. Ammonium in the evaporated samples was measured. Ammonia was determined by a slight modification of the micro diffusion technique. A round-ended glass rod which passed through a rubber stopper was dipped into concentrated H_2SO_4 and the excess was removed by touching a paper towel. A 200-pL sample (enzyme and tissue extracts, or standards containing 0-800 nmol ammonium N). 100 uL sample was added into a 20 mL scintillation vial containing 1 mL saturated K_2CO_3, followed by rapid seating of the rubber stopper and gentle agitation of the vial. After 60 min standing at room temperature, the stopper was removed and the glass rod used to stir 1.5 mL of Nessler's reagent (1 mL H_2O : 0.5 mL Nessler's reagent held in a 1.5 mL microtube, then was capped and incubated for 5 min at room temperature before reading the absorbance at 420 nm. Nitrate content was measured according to a method of [10], water extracts (usually 0.2 mL) are taken into 50 mL Erlenmeyer flasks, thoroughly mixed with 0.8 mL of 5% (w/v) salicylic acid in concentrated sulfuric acid (SA-H_2SO_4), and allowed to settle for 20 min at room temperature. Then 19 mL of 2 M sodium hydroxide (NaOH) was slowly added to raise the pH above 12. Finally, the samples

were cooled to room temperature, and the absorbance at 410 nm was determined.

Immunoblot Analysis. The immunoblot analysis was optimized by preliminary experiments. After electrophoresis, the proteins were electrotransferred onto a nitrocellulose membrane (Hybond-ECL, Amersham Biosciences, Germany) at 2 mA/cm^2 for 60 min using a Bio-Rad Trans-Blot semidry electroblot system (Bio-Rad Laboratories, Hercules, CA) and a Tris-glycine transfer buffer system (25 mM Tris, 192 mM glycine, 0.1% SDS, and 10% MeOH). The membrane was washed three times with Tris-buffered saline (25 mM Tris-HCl, 137 mM NaCl, and 2.68 mM KCl, pH 7.4) containing 0.05% Tween 20 (TBS-T) and then blocked with 1% polyvinylpyrrolidone in TBS-T for 4 h at room temperature. After it was washed three times with TBST, the membrane was incubated overnight with primary antibody diluted 1:50 with TBS-T at room temperature. The mixed patients sera were prepared by mixing equal volumes of sera from all seven patients. The membranes were washed three times with TBS-T and then incubated with Alkaline-phosphatase conjugated goat anti-rabbit antibody (American Qualex, San Clemente, CA) at a dilution of 1:2000 for 2 h at room temperature. Blots were then washed three times in TBS-T before visualization. Antibody complexes captured by the immobilized target protein are detected by an enhanced HRP detection system (Amersham Biosciences, United Kingdom); in the presence of H_2O_2, HRP converted luminol to an exciting intermediate dianion that emitted blue light (428 nm) on return to the ground state [11].

3. Results and Discussion

The activity of NR and GS on the different sources of nitrogen.

Nitrogen is an essential nutrient for the formation of nucleotides and proteins. It is the nutrient that most limits the yield of rice. Seedling seeds require ammonium and nitrate; therefore, ammonium-based fertilizers such as ammonium sulfate and urea are widely used in rice cultivation. The addition of nitrate to the rice plant media caused an increase in nitrate reductase activity, but its activity decreased after the addition of more than 6.4 nm. Although both forms of nitrogen could increase NR activity, it was higher in media containing nitrate. Nitrates are needed by plants to induce the formation of the NR gene. Nitrate stimulates the expression of the NR gene at the transcription level, thus increasing the expression of mRNA and the synthesis of NR proteins [12].

Nitrate reductase activity was higher in all nitrate treatment variations than ammonium treatment. NR in vascular plants is a substrate inducible enzyme [6] and NO^{-3} is the primary factor regulating NR induction [13]. In all cases, nitrate concentration is a factor that regulates the induction of NR [4]. The higher the substrate concentration (nitrate), the higher the NR activity, even at the highest nitrate concentration of 12.8 mM in this study. The increase in NR activity to the highest nitrate concentration indicates that the plant has not reached its capability to accumulate nitrates or NR products, since nitrate accumulation or NR activity products have a negative correlation with NR activity [14]. Increased NR activity due to nitrate induction is also shown in another study in rice [15], maize [16], tomato [17], and soybean [18]. In addition to being a source of N in many plants, nitrate also plays a role in signaling for the initiation of multiple processes. Carbohydrate metabolism is also influenced by the presence of nitrate in the synthesis of starch and sucrose desired plant [19] then can support the needs of NADH and NADPH as electron donors through photosynthesis and respiration process.

Ammonium was not a NR substrate and did not directly affect NR activity, ammonium is not needed in enzyme synthesis directly, but it leads to increase protein synthesis in general and synthesis of the effector molecule that important for NR activity. Therefore ammonium treatment takes a long time to improve NR activity. In addition to improving the NR activity, ammonium and amino acids such as glutamine and asparagine can be the most potent inhibitors in NR induction [20]. The ammonium content in the cells will lead to the formation of more amino acids. Moreover, high concentrations of ammonium ions in plants can inhibit the absorption of nitrate as an NR substrate and decrease its activity [21].

The result of the glutamine synthetase (GS) activity test shows that nitrate and ammonium treatment can increase GS activity until a certain concentration and at higher concentration have negative relation. In the nitrate treatment, optimal GS activity at 1.6 mM nitrate concentration, after which showed a decrease and steady at concentrations of 6.4 mM to 12.8 mM. In ammonium treatment, the optimum GS activity was in a concentration of 3.2 mM ammonium, after which showed a decrease and steady in concentrations of 6.4 mM to 12.8 mM ammonium. Treatment both of nitrate and ammonium at concentrations of 3.2 mM showed relatively similar GS activity. In addition to being a source of N in many plants, nitrate also plays a role in signaling for the initiation of multiple processes. Carbohydrate metabolism is also affected by the presence of nitrates in the synthesis of starch and sucrose desired by plants which will produce organic acids such as oxoglutarate and act as an acceptor to reduce N in the GS-GOGAT cycle [22]. GS and GOGAT activity increased at low external nitrate concentrations and decreased at high cytosolic nitrate concentrations [23]. On Ciherang rice varieties, the optimal concentration of nitrate is 1.6 mM. The decrease in GS and GOGAT activity at high nitrate concentrations leads to a decrease in the accumulation of ami-

no acids produced in the leaves, thus decreasing the activity of GS [24]. In plants supplied with ammonium, ammonium will be transported by the transporter and then assimilated to glutamine by GS-GOGAT resulting in the accumulation of amino acid in the cell. This indicates that ammonium can restore GS-GOGAT activity and increase the accumulation of amino acids in the leaves [25][26]. The results of this study indicate that nitrate and ammonium can increase GS activity, but ammonium treatment in different concentration show more stable activity than nitrate treatment. Furthermore, the decrease in GS activity at high ammonium treatment may be the way plants limit the excess supply of glutamine, thereby reducing the amount of cellular carbon.

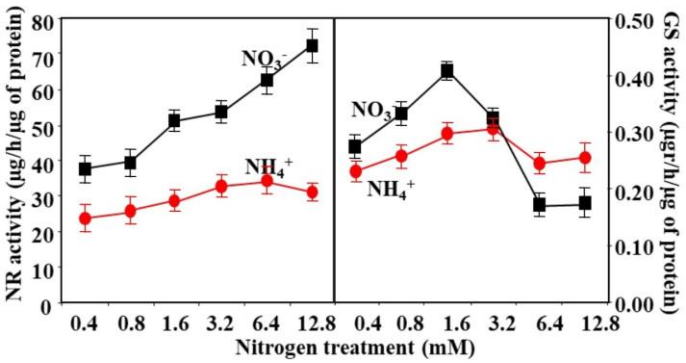

Figure 1. NR and GS activities in rice leaf different treatment of nitrogen.

Western blot analysis showed that the GS2 protein bands increased with the addition of ammonium concentrations. GS2 protein has a molecular weight of 40.5 kD, indicating the intensity of the darkest in the delivery of ammonium concentrations of up to 3.2 mM. The concentration of 0.4 mM showed more intensity light than higher concentrations. This suggests that increasing the content of GS2 was affected by increasing concentrations of ammonium (Figure 2A). The content of GS2 tended to decrease in intensity during nitrate treatment. Increasing the content occurred from 0.4 mM concentrations up to 1.6 mM, and then tended to decline at a concentration of 3.2 up to 12.8 mM. The availability of ammonium affected the GS2 protein content greater than nitrate, GS2 protein was regulated by the presence of ammonium.

GS2 was the ammonium assimilation enzyme that catalyzes the synthesis of glutamine from ammonia so that it has a very great affinity for ammonia. GS2 has an important role in the assimilation of nitrogen and was located in the chloroplast. Nitrogen is the material basis for many biochemical and physiological functions [27], such as the formation of amino acids, proteins, purine bases, pyrimidine bases, nucleic acids, and other essential compounds. A high concentration of ammonium leads to increased activity of glutamine synthase formed glutamine. Glutamine was a precursor for the formation of amino acids so that most of the proteins were affected by the concentration of glutamine. At the stage of translational protein synthesis occurred, GS2 activity was inducted by the presence of light. light intensity caused the increase of GS2 protein synthesis, so the GS2 regulation occurred at the translational and post-translational stages. The stability of transcriptional regulation of GS2 has an important role in rice development and in carbon-nitrogen metabolic balance. A low level of GS2 expression caused an imbalance of carbon-nitrogen metabolism and was the reason chlorosis occurs[28]. nitrogen assimilation and recycling of young leaves occur in chloroplasts, as well as nitrite reduction and ammonium assimilation (performed by GS or GOGAT). This process involves GS2 and fdGOGAT, therefore the nitrogen treatment in the young rice phase can increase the activity of both GS2 and GOGAT genes. Meanwhile, chloroplast discharges in the aging process involve another GS isomer GS1[29].

The intensity of the GS1 protein band increased with the increasing concentration of ammonium. The 0.4 mM ammonium concentrations up to 1.6 mM showed the same intensity tends. Increased intensity occurred at a concentration of 3.2 mM up to 12.8 mM ammonium. The content of GS1 increased with increasing concentrations of ammonium, resulting in the formation or increase in protein synthesis. Ammonium given to rice crops in the early growth led to an increase in protein synthesis GS1. Nitrate gave the opposite effect to the GS1 protein content (Figure 2B). The higher nitrate concentrations caused a decrease in the content of GS1. The protein content of nitrate 0.4 mM up to 3.2 mM tends to be the same and then decreased with increasing nitrate concentrations up to 12.8 mM (Figure 2B). Or ammonium nitrate concentrations were increased not affect GS1 protein content (Figure 2B). GS1 is an enzyme present in the cytosol and implies very little influence by the concentration of nitrate or ammonium. GS1 is one of the GS isoenzymes that are not affected by the presence of light. GS1 related to ammonium recycling during some plant development processes such as leaf senescence, GS1 also had a role in the glutamine synthesis i.e transporting to phloem sap [30]. GS1 is a more complex protein, consists of several isoforms encoded by a multigenic family, and there are three genes encoded GS1 in rice: *OsGS1;1*, *OsGS1;2* and *OsGS1;3*. *OsGS1;1 was* expressed in all organs and has functions in translocating nitrogenous compounds to the developing sink tissues [31] stated that *OsGS1;2* was detected also in all organs, higher expressed in the root at the seedling process, and had the main function to assimilated NH_4^+. *OsGS1;3* found in spikelet [31] and not much discussed, [32] stated that mutant OsGS1;3 showed reduce in natural senescence of the rice field, and showed a slow rate of germination. These genes are not regulated in the same way and located in the different a part of plant and do not have the same kinetic properties

[29] so that its expression is not directly influenced by the nitrogen content, it may be influenced by more than any other factor since each isoform has distinctive features and regulation. [33] reported that nitrate induced the accumulation of GS1-5 while ammonium induced the accumulation of both GS1-1 and GS1-5 in maize.

The content of GOGAT enzyme was shown nearly the same intensity. The content GOGAT not influenced by the concentration of nitrogen (figure 3). The intensity of the protein GOGAT has the same tendency as the provision of ammonium or nitrate at different concentrations. The content of GOGAT more affected by ferredoxin as an electron donor of pyrimidine in nucleotides [34]. Increasing nitrogen concentrations do not affect the content of GOGAT and glutamate produced due GOGAT gene expression is affected by light. Light will solve ferredoxin as electron donors for the assimilation of ammonium in the chloroplast.

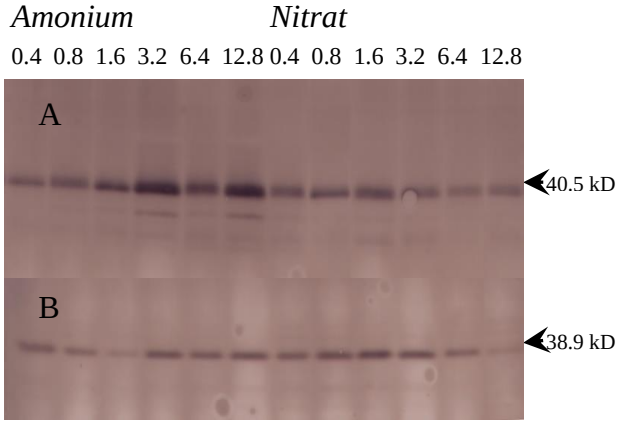

Figure 2. Western blot analysis of GS2 dan GS1 in leaf with different nitrogen treatment.

Ammonium and nitrate accumulation in leaf

Ammonium content of leaf tissue increased in the media contained ammonium (Figure 3A). Rice leaf accumulated a little amount of ammonium and temporary storing in the tissue then rapidly assimilated into glutamine catalyzed by ammonium assimilation enzymes (GS and GOGAT). The accumulation of nitrate in rice leaf tissue showed that it was an accumulation over 100 times larger than ammonium (Figure 3A and 3B). Tissue ammonium content showed an increasing trend with increasing nitrogen treatment in the form of ammonium although the increase in the compounds did not show a significant difference in each treatment. Nitrates give no real effect on rice plants that do not show an increase in nitrate content despite improved treatment, except in nitrate concentration of 1.6 mM. The content of nitrate increased dramatically at a concentration of 1.6 mM showed that the nitrate concentration of 1.6 mM is suitable for rice plants to make the process of assimilation so that the accumulation of ammonium is greater than the treatment less than or greater than 1.6 mM. Rice crop requires very low nitrate so natural that an increase in the concentration of 1.6 mM because at less than 1.6 mM concentration is too low and the concentration of more than 1.6 mM is too high for rice crops to the assimilation of nitrogen.

Nitrate accumulation in leaves of rice plants increased with increasing nitrogen applications in the form of ammonium or nitrate. Provision of nitrogen in the form of nitrate or ammonium nitrate accumulation tends to increase although it did not show significant differences in each treatment. The content of nitrate in the plant tissue of rice is very large compared to ammonium. Rice plants absorb nitrogen in the form of nitrate or ammonium (Figure 5B). Nitrogen is absorbed in the form of nitrate accumulated in the vacuole. Accumulation of nitrate in leaf tissue enhanced with increasing concentrations of nitrate or ammonium. This is supported by the increased activity of NR with increased concentrations of nitrogen (Figure 4A).

The rice plant demonstrated its ability to assimilate nitrogen in the form of nitrate and ammonium for adaptation to environmental conditions. Adaptation of the rice plant to sources of nitrate or ammonium could increase protein synthesis of NR at the transcriptional and translational levels that the activity against both sources of nitrogen are very high [35]. The rice plants can adapt and grow well in nitrate or ammonium as the formation of NR genes was identified as NR and NR the specific NADH-NAD (P) H-bispesific. NR is a key enzyme in the assimilation of nitrogen in rice although many reports stated that the rice absorbs nitrogen in the ammonium form.

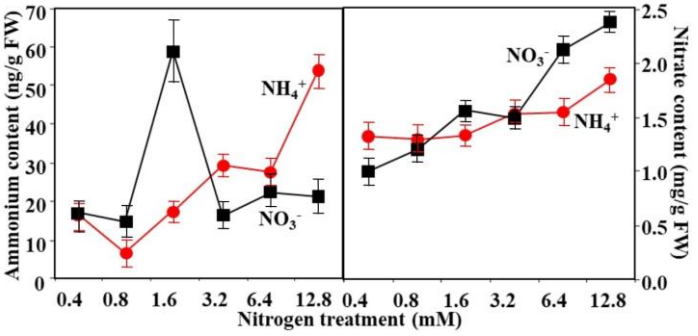

Figure 3. Ammonium and nitrate content in rice leaf with different treatment of nitrogen.

4. Conclusion

The available source of N for rice was in nitrate form, easily uptake by the rice plants during the growth stage. Nitrate in-creased NR and GS activity in media contained nitrate. Addition of ammonium in level in 3.2 mM induced in ammonium content in leaves.

5. Acknowledgement

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