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Measurement of Nitrate Reductase Activity in Vivo Using Sepctrophotometry Method

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ABSTRACT

Spectrophotometry is a quantitative measurement technique of a compound based on the measurement of absorbance or light transmission passing through the compound. The spectrophotometric method can be used to analyze the content of compounds. One of the enzymes whose activity can be measured in vivo is the nitrate reductase enzyme. The nitrate reductase enzyme is an enzyme that plays a role in the synthesis of ammonium and amino acids. The results of measuring the activity of the nitrite reductase enzyme with spectrophotometry showed that the highest NRA value was found in the leaves of red chili plants and the lowest was in the sugarcane leaf sample. NRA can be used as a selection parameter to predict the results of a plant, and has the potential to be applied to seed plants.

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INTRODUCTION

Spectrophotometry is a quantitative measurement technique of a compound based on the absorbance or transmission of light passing through the compound (Germer *et al*., 2014). Spectrophotometry consists of several types based on the light source used. Among them is visible spectrophotometry (Vis), which uses visible light with an electromagnetic spectrum that can be perceived by the human eye. Unlike visible light spectrophotometry, UV spectrophotometry is based on the interaction of the sample with UV light. Other types of spectrophotometry include UV-Vis spectrophotometry and infrared spectrophotometry. UV-Vis spectrophotometry uses two different light sources: UV light and visible light. Some spectrophotometers use only one light source for both UV and Vis, namely a photodiode equipped with a monochromator. Meanwhile, infrared spectrophotometry is based on the absorption of infrared wavelengths. Infrared light is divided into near-infrared, mid-infrared, and far-infrared**.**

The spectrophotometric method can be used to analyze the composition of compounds, measure bacterial growth, and assess enzyme activity (Germer *et al*., 2014). One enzyme that can be measured in vivo is nitrate reductase. This enzyme plays a role in the synthesis of ammonium and amino acids by reducing nitrate to nitrite. Nitrate reductase transports two electrons from NADH or NADPH, resulting in nitrite (NO_2-), NAD+, and H_2O**.** This reduction occurs in the cytosol outside cellular organelles. Nitrate reductase consists of two identical polypeptide subunits and contains FAD, iron in the heme prosthetic group, and molybdenum**,** all of which are reduced and oxidized in coordination with the electron transfer from NADH to the nitrogen atom in NO_3-. The activity of nitrate reductase often influences the rate of protein synthesis in plants, as NO3- is absorbed as the primary nitrogen source (Lakitan, 2000).

Plants absorb nitrogen in the form of nitrate, ammonium salts, and organic compounds. The use of nitrogen in the form of nitrate requires a nitrate reduction process**,** where nitrite is formed as an intermediate product before being converted into ammonia as the final product. Nitrate reduction occurs in darkness**,** but according to Peni *et al*. (2004), nitrate reduction in green leaves can be accelerated in light conditions, allowing both nitrate and CO_2 to be reduced in the dark phase of photosynthesis. NR activity measurement is necessary as NR activity often affects the rate of protein synthesis in plants that absorb NO3- as the main nitrogen source (Lakitan, 2000).

The activity of nitrate reductase enzyme (NRA) is known to have a positive correlation with crop yield in various plants, such as corn, tea, and coconut**.** This presents a promising opportunity for selecting high-yielding crops**.** Rockel *et al*. (2002) stated that NRA can be used as a selection parameter to estimate plant yield. In wheat plants, NRA as a selection parameter can be applied even at the seedling stage.

MATERIALS AND METHOD

Materials

The plant materials used include 1 gram of leaves from soybean, peanut, chili, sugarcane, and corn plants. The chemical reagents used include 5M NaNO3 as a substrate, 0.6M NaNO2 as a standard solution, 0.1M phosphate buffer (pH 7.5) as a buffer solution, 0.02% napthylediamine (NED) as a solution that binds diazonium ions, 1% sulfanilaminde (SA) as a color indicator, 3N HCl, and distilled water as a diluent.

Methods

1. In Vivo NRA

Each plant leaf sample was cleaned from dust and water using tissue paper. The midrib was separated from the lamina and discarded. 1 gram of plant leaves was weighed using a semi analytical balance, then sliced into 2 mm thick pieces. Each plant leaf sample was tested in duplicate. The leaf slices were placed into a dark tube (film tube) containing 5 mL of phosphate buffer (pH 7.5) and soaked for 15 minutes. The phosphate buffer was then discarded and replaced with a fresh 5 mL buffer. After that, 100 µL of 5M NaNO3 was added the tube and incubated for 30 and 60 minutes.

During incubation, test tubes were prepared, each containing 200 µL of 0.02% NED solution and 200 µL of 1% SA solution in 3N HCl. After the incubation period, 100 µL of the leaf extract solution was transferred into the prepared test tube containing NED and SA. After 10 minutes of absorbance was the measured using a visible spectrophotometer at a wavelength of 540 nm.

2. *Preparation of the nitrite (NO_2^-) standard curve*

To prepare the standard solution, 60 µM NaNO3 was measured and distributed into eight test tubes. Each tube was filled with solutions as specified in **Table 1**. The solutions in each test tube were then vortexed and their absorbance values measured using a visible spectrophotometer. A graph was then created to represent the relationship between nitrite concentration and absorbance values, followed by the formulation of a linear equation.

3. *Determination of In Vivo NRA Values*

The in vivo (Nitrate Reductase Activity) value was deterimed using the following formula:

$$\mu mol\ NO_2^- \times \frac{5\ mL}{0.1\ mL} \times \frac{1000\ mg}{1000\ mg} \times \frac{60\ minutes}{incubation\ time\ (minute)}$$

Table 1. Composition for Nitrite Standard Curve Determination

Solution	Test Tube Number							
	1	2	3	4	5	6	7	8
NO_2^- (nmol)	0	4	8	12	16	20	24	28
60 µM $NaNO_2$ (µL)	0	100	200	300	400	500	600	700
NED (µL)	200	200	200	200	200	200	200	200
SA (µL)	200	200	200	200	200	200	200	200
Distilled Water (µL)	2600	2500	2400	2300	2200	2100	2000	1900
Total Volume (µL)	3000	3000	3000	3000	3000	3000	3000	3000

RESULTS AND DISCUSSION

NRA Measurement

The NRA value measurements for six plant leaf samples are shown in **Table 2.**

Table 2. Measurement value of NRA for six plant leaf

Samples	Absorbance			Concentration			NRA			Average
	1	2	3	1	2	3	1	2	3	
Cayenne paper 30’	0,098	0,048	0,064	2,683	1,116	1,618	268,34	111,60	161,76	180,56
Cayenne paper 60’	0,192	0,118	0,139	5,630	3,310	3,969	281,50	165,52	198,43	215,15
Red chili paper 30’	0,541	0,326	0,238	16,571	9,831	7,072	1657,05	983,07	707,21	1115,78
Red chili paper 60’	0,778	0,773	0,609	24,000	23,843	18,702	1200,00	1192,16	935,11	1109,09
Peanut 30’	0,051	0,046	0,132	1,210	1,053	3,749	121,00	105,33	374,92	200,42
Peanut 60’	0,048	0,045	0,071	1,116	1,022	1,837	55,80	51,10	91,85	66,25
Rice 30’	0,026	0,009	0,01	0,426	-0,107	-0,075	42,63	-10,66	-7,52	8,15
Rice 30’	0,017	0,015	0,02	0,144	0,082	0,238	7,21	4,08	11,91	7,73
Sugarcane 30’	0,008	0,058	0,009	-0,138	1,429	-0,107	-13,79	142,95	-10,66	39,50
Sugarcane 60’	0,03	0,033	0,026	0,552	0,646	0,426	27,59	32,29	21,32	27,06

Maize 30’	0,104	0,093	0,106	2,871	2,527	2,934	287,15	252,66	293,42	277,74
Maize 60’	0,268	0,252	0,209	8,013	7,511	6,163	400,63	375,55	308,15	361,44

Nitrite Standard Curve

The nitrite standard curve is obtained from the absorbance measurements of each nitrite concentration, as shown in **Table 3.**

Table 3. Absorbance Measurements of Each Nitrite Concentration

Conc. of nitrite (nM)	Absorbance λ540 nm					
	1	**2**	**3**	**4**	**5**	**6**
0	0,013	0,02	0,079	0,015	0,039	0,065
4	0,128	0,24	0,225	0,149	0,14	0,187
8	0,226	0,255	0,345	0,259	0,269	0,212
12	0,346	0,367	0,419	0,395	0,343	0,541
16	0,395	0,502	0,627	0,53	0,534	0,535
20	0,692	0,667	0,845	0,633	0,634	0,579
24	0,797	0,735	0,854	0,767	0,782	0,805
28	0,948	0,887	0,944	0,926	1,022	0,944
R value	0,9742	0,9831	0,9745	0,9985	0,9824	0,953

From the measurement data above, the fourth data set is considered the most suitable for constructing the standard curve. The nitrite standard curve is shown in **Figure 1**.

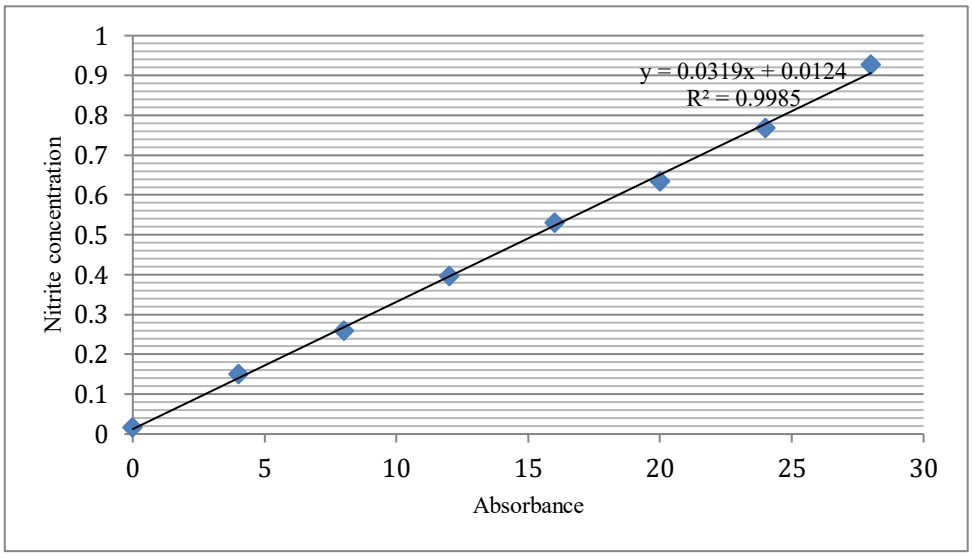

Figure 1. Nitrite curve standard

This study tested nitrate reductase activity in six plant leaf samples, namely bird's eye chili, red chili pepper, soybean, peanut, sugarcane, and corn leaves. The results of nitrate reductase activity measurements for each plant sample showed different values depending on the incubation duration. The incubation times used in this experiment were 30 minutes and 60 minutes. The experimental results showed that the highest ANR value was found in red chili pepper leaves with a 30-minute incubation time, which was 1115.78 µmol NO2 / gram leaf weight / hour of incubation. Meanwhile, the lowest total ANR value was found in sugarcane leaf samples, at 7.73 µmol NO2 / gram leaf weight / hour of incubation for 60 minutes.

The difference in productivity among the six plant samples can be determined from the obtained ANR values. This aligns with the study by Sadikin (2002), which states that the relationship between reaction rate and enzyme activity is directly proportional. The higher the enzyme activity, the faster the reaction rate. A faster reaction rate results in more product formation. This proportional relationship can support plants in increasing their productivity. Therefore, if nitrate reductase activity increases, the resulting product will also increase along with the reaction rate.

The average ANR value for the six plant leaf samples incubated for 30 minutes was higher than for those incubated for 60 minutes. However, in the case of bird's eye chili leaves, the highest ANR value was observed at 60 minutes of incubation (215.15), while for sugarcane leaves, it was 39.50 at 60 minutes. This is consistent with the study by Rockel *et al.* (2002), which states that nitrate reductase activity is influenced by several factors, including the rate of synthesis and degradation of enzymes by proteinases. Nitrate reductase activity degrades in the dark reaction with a decline in activity. After this phase ends, proteolysis occurs, and proteinase enzymes degrade nitrate reductase activity. Meanwhile, according to Peni et al. (2004), nitrate reductase activity is influenced by many factors, including the amount of light absorbed by the leaves, soil pH, temperature, humidity, plant age, inhibitors (such as tannins and phenols), and substrate availability.

According to Peni *et al.* (2004), nitrate reduction in green leaves can be accelerated under light conditions, allowing both nitrate and CO2 to be reduced in the dark reaction of photosynthesis. The samples were placed in dark tubes to ensure nitrate was reduced by the dark reaction with the help of nitrate reductase enzyme until only nitrite remained. However, if exposed to sunlight, nitrate will be reduced to ammonia. Most higher plant species can reduce nitrate to the ammonia stage.

This study used a 5M substrate concentration without variation. Substrate variation can be conducted to determine the inducible nature of nitrate reductase, meaning its activity increases with substrate addition (Rockel *et al*., 2002). In this experiment, a coloring reagent such as NED solution was also used. The addition of NED solution functions to produce a color that indicates the nitrite content. Nitrite is a product of the nitrate reductase enzyme reaction. The prepared nitrite solution was then visualized in a linear graph. Based on the obtained nitrite solution results, the fourth dataset was considered the best for constructing the standard curve, with a regression value (R2) of 0.9985. The standard curve formed was excellent. This aligns with Saakov et al. (2013), who stated that a standard curve is considered good or acceptable if its regression value (R2) is ≥ 0.999 or close to 1. If the regression value (R2) is greater than 1 (too high), the sample solution must be diluted. If the regression value (R2) is much lower than 1, the sample solution needs to be remade or further diluted.

Based on ANR measurements, some nitrite concentration absorbance data did not show data linearity and had varying R2 values. However, the average R value was close to 1. This variation may have been caused by measurement inaccuracies, leading to an R value that did not reach 1. The obtained standard curve results allowed a comparison of ANR values for each sample and incubation time.

For bird's eye chili leaf samples incubated for 30 minutes, the absorbance value in the first repetition was 0.098, which did not match the standard curve. The second and third repetitions showed similar trends with values of 0.048 and 0.064, respectively.

CONCLUSION

The highest ANR value was found in the leaves of red chili plants with an incubation time of 30 minutes, which was 378.73 µmol NO2/gram leaf weight/hour of incubation. Meanwhile, the lowest total ANR value was found in the sugarcane leaf sample, which was 56.76 µmol NO2/gram leaf weight/hour of incubation. The ANR absorbance value that was closest to the standard curve value was the first repetition absorbance of the red chili leaf incubated for 60 minutes, which was 0.778, while the ANR absorbance value farthest from the standard curve was the first repetition absorbance of the sugarcane leaf incubated for 30 minutes, which was 0.008.

AUTHORS CONTRIBUTION

W.M. designed and conducted the research, analysed and interpretation the data, and wrote the draft of manuscript, S.W & D. M. analysed and intrepetation the data.

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