NITROGEN USE EFFICIENCY AND $^{13}$C ISOTOPE DISCRIMINATION IN NMR151 AND NMR152 RICE MUTANT LINES UNDER DIFFERENT WATER POTENTIALS AND NITROGEN RATES

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ABSTRACT

This study was conducted to evaluate the nitrogen use efficiency and $^{13}$C isotope discrimination of rice mutant lines viz. NMR151 and NMR152. Both cultivars are developed under rice radiation mutagenesis programme for adaptability to aerobic conditions. In the present study, NMR151 and NMR152 were grown under conditions of varying water potentials and nitrogen levels in a shade house. The rice mutant lines were planted on sandy loam soil. Three watering regimes and three nitrogen levels in a completely randomized design with three replications were carried out. The rice mutants were grown for 110 days under three water potentials, (i) Field capacity from 0 to 40 DAS and saturated from 41 to 110 DAS [ST], (ii) Field capacity from 0 to 110 DAS [FC], and (iii) Field capacity from 0 to 40 DAS and 70% of field capacity from 41 to 110 DAS [SS]. Direct $^{15}$N isotopic tracer method was used in this study, whereby the $^{15}$N labelled urea fertilizer 5.20% atom excess (a.e) was utilized as a tracer for nitrogen use efficiency study (NUE) by the test crops. $^{15}$N isotope presence in the samples was determined using emission spectrometry and percentage of total nitrogen was determined by the Kjeldahl method. $^{15}$N a.e values of the samples were used in the determination of the efficiency of N used by the mutant varieties. The $^{13}$C isotope discrimination ($\Delta^{13}\text{C}$) technique was used as a tool to identify drought resistance rice species with improves water use efficiency (WUE). WUE is the ratio of the biomass produced by the water consumed. For $^{13}$C discrimination analysis, a sample of rice leaf was analyzed for $^{13}$C content by using Isotope Ratio Mass Spectrometer (IRMS). The parameter, viz. plant height, number of tillers, grain yield, 1000 grain weight, NUE, $\Delta^{13}$C and WUE were recorded. Results from this study showed nitrogen rates imparted significant effects on plant height, number of tillers, grain yield, 1000 grain weight and WUE, while water potentials had significant effects on plant height, grain yield, 1000 grain weight, NUE, $\Delta^{13}$C and WUE. $\Delta^{13}$C has a low but significantly negative relationship with WUE. Rice mutants NMR151 and NMR152 were found to be not significantly different except for their NUE.

Keywords: $^{13}$C isotope discrimination, $^{15}$N isotopic tracer, nitrogen use efficiency, rice mutant lines, water potentials, water use efficiency

INTRODUCTION

As a major contributor to the world's staple food, the rice sector is also the largest user of water. Lack of water is a major concern in this sector. The main method of rice cultivation in Malaysia is through flooding. This type of cultivation consumes very high amount of water, where about 3000 L of water are used to produce 1 kg of rice (Sariam et al., 2002). Over 80% of the freshwater resources in Asia are used for irrigation and about half the amount is consumed for rice (Dawe et al., 1998). Irrigation becomes increasingly vulnerable to competition on available water resources due to population rate remaining high and other (non-irrigated) put pressure on limited water
resources, especially during summer or drought season. For strategic water management and utilization purposes the traditional submerged rice cultivation system is gradually moving towards a system where rice is grown under low water input and aerobic conditions, while maintaining high productivity.

Aerobic cultivation is a planting system where rice is grown in well-drained, non-puddled, and non-saturated soils. In aerobic rice systems, fields remain unsaturated throughout the season. Yields are on average at 1 to 2 t ha\(^{-1}\) because of adverse environmental conditions (poor soils, little rainfall, weeds), low use of external inputs, and low yield potential of upland rice cultivars (Priyanka et al., 2012). Generally, rice is grown in the fertile highlands using high yielding cultivars with adequate water supply also, can be considered as aerobic rice (Kato et al., 2009). The previous report showed that aerobic rice used only 50% of the water as compared to lowland rice (Sheng et al., 2012). Aerobic cultivation can reduce the total water usage by 27 to 51% and increase water productivity by 32 to 88% (Bouman, 2005).

Nitrogen (N) is one of the essential macronutrients for rice growth and one of the main factors to be considered for developing high-yielding rice cultivar (Duan et al., 2007). N requirements of the rice plant are supplied from soil and fertilizer. Due to the acute shortage of N in the soil mostly, N fertilizer must be used to meet the needs of the plant. In flooded rice ecosystems, some reports said that N fertilizer used for rice crops were lost partly through different mechanisms including ammonia volatilization, denitrification, leaching and run by water (Choudhury and Kennedy, 2005; Dobermann and Fairhurst, 2000). Loss of nitrogen can cause environmental problems such as pollution of the atmosphere, aquatic systems and ground water in a bowl.

Nitrogen use efficiency (NUE) is generally low with normally less than 30% to 40% of the applied N taken up by flooded rice (Abou Seeda et al., 1994; Craswell and Vlek, 1979; De Datta, 1986). This situation challenges the researchers to develop the strategies to increase NUE in rice production. Increasing of NUE offers the greatest economic benefits to farmers and countries. NUE may be defined as yield per unit input. In agriculture this is usually related to the input of N fertilizer, whereas in scientific literature the NUE is often expressed as fresh weight or product yield per content of nitrogen (Janssen, 1998). NUE of crops can be improved by adopting adequate management practices. Use of N fertilizers in adequate amount, form and methods of application are important management strategies of this element (Tayefe et al., 2011).

To overcome the water scarcity problem, many researchers including in Malaysia are trying to generate new rice cultivars suitable for planting under aerobic conditions. Malaysian Nuclear Agency (Nuclear Malaysia), in collaboration with the Malaysian Agricultural Research and Development Institute (MARDI) and Universiti Putra Malaysia (UPM), generated mutant lines of rice viz. NMR151 and NMR152. Both cultivars have been developed under the mutagenesis program for adaptation to aerobic conditions. The parent plant of these cultivars is the MR219 from MARDI. The parent seed was exposed to gamma irradiation at 300 to 400 Gy. These new cultivars can be grown under minimal water input (Abul Rahim and Abdullah, 2012).

The only direct means of measuring NUE from the applied fertilizer is through the use of isotopes. In NUE study, a labelled fertilizer \(^{15}\text{N}\) is added to the soil and the amount of nitrogen that a plant has taken up is determined. In this way, different N fertilizer practices (placement, timing, and sources) can be studied (IAEA, 2001). Isotopic studies using \(^{15}\text{N}\) labelled plant materials have been useful in estimating crop N uptake (Azam et al., 1993; Jensen, 1994; Vanlauwe et al., 1996).
The $^{13}$C isotope discrimination technique has become a powerful research tool to identify and select $C_3$ and $C_4$ plant species (eg: rice, wheat, and maize) with improving water use efficiency (WUE) (Dercon et al., 2006). WUE is the ratio of the biomass produced by the water consumed. For $C_3$ plants, Farquhar et al. (1982) proposed that $^{13}$C discrimination ($\Delta^{13}$C) is related to diffusional fractionation ($a = 4.4 \%$) and discrimination against $^{13}$CO$_2$ by ribulose diphosphate carboxylase or RuBisCo ($b = 30 \%$), and $Ci/Ca$, the ratio of intercellular to ambient partial pressure of CO$_2$. It can be expressed as equation below.

$$\Delta C_3 = a + b \left( b - a \right) \frac{Ci}{Ca}$$

While the fractionation parameters $a$ and $b$ are constant, the $Ci/Ca$ ratio is controlled by genetics factors and environmental constraints. Environmental factors such as drought, soil compaction and increased vapour pressure deficit are known to have an effect on $^{13}$C isotope discrimination (Dercon et al., 2006). When for instance soil moisture level decreases, plant close the stomata to reduce water loss and the CO$_2$ diffusion from the air outside to the air inside the leaf will be reduced. Based on equation 1, it is shown that this reduction of CO$_2$ concentration inside the leaf will cause the decrease of $\Delta^{13}$C or a higher $^{13}$C abundance of the fixed CO$_2$ (Dercon et al., 2006). In other words, for $C_3$ plants, the increasing of water stress will cause reducing of $\Delta^{13}$C.

The application in the field to assess water stress is hampered by other common stress factors such as nutrient deficiency (Clay et al., 2001; Shangguan et al., 2000). The $^{13}$C isotope discrimination is reduced by the increase of water stress and decrease of nitrogen level in the soil (Shangguan et al., 2000). Water use efficiency was negatively correlated with $\Delta^{13}$C, so WUE was increased with N supply and with increasing water stress (Farquhar and Richards, 1984).

The objective of this study is to evaluate the nitrogen use efficiency (NUE) of NMR151 and NMR152 and to assess the potential use of $^{13}$C discrimination observation to quantify water stress and it dynamics under different water potentials and different N rates. The interaction between N levels and water potentials will also be reported in this study.

**MATERIALS AND METHODS**

**Experimental Design**

A shade house experiment was carried out in a paddy shade house in Malaysian Nuclear Agency. The shade house provided some control on environmental factors, including shelter from the rain. Two types of mutant lines rice varieties namely NMR151 and NMR152 were used in this experiment. Both varieties are produced by mutation induction using gamma irradiation on MR219 variety (parent). Mutation induction was carried out by exposure of plant organs such as seeds or biological entity to ionizing radiation that can penetrate the tissue cells so that the heritage elements of the chromosome and the process causes the physical changes to the plants (Abdul Rahim and Abdullah, 2012). The soil used in this experiment is a loamy texture with a bulk density of 1.45 g/cm$^3$. Water retention curve analysis of this soil has been done using pressure plate method. The curve showed that available water capacity calculated between 10 to 1500 kPa for this soil is about 18% (w/w). Completely Randomized Designed (CRD) will be used as experimental layout.
Rice Planting

Rice mutant line NMR151 and NMR152 were planted on sandy loam soil in troughs measuring 3 m² (3m x 1m). Rice seeds are sown manually at 2 cm depth and covered with soil. Three watering regimes and three N rates in a completely randomized design with three replications were carried out. The rice were grown for 110 days under three water potentials, (i) Field capacity from 0 to 40 DAS (days after sowing) and saturated from 41 to 110 DAS [ST], (ii) Field capacity from 0 to 110 DAS [FC], and (iii) Field capacity from 0 to 40 DAS and 30% dry of field capacity from 41 to 110 DAS [SS]. Nitrogen in the form of 15N-labeled urea (5.20% atom excess) was applied at three rates; none (0 kg N/ha), moderate (60 kg N/ha) and high (120 kg N/ha) in three splits at 7 days after emergence, 35 days after emergence and 60 days after emergence. Basal fertilizers of phosphorus from triple super phosphate (TSP) at 60 kg P₂O₅/ha and potassium from muriate of potash (MOP) at 60 kg K₂O/ha were applied at 15 days and 65 days after emergence. A tensiometer, an instrument that directly measures soil moisture continuously was installed. Soil moisture was measured daily.

Water Usage

Water meters were installed with irrigation systems for measuring water usage at planting season for assessment of water use efficiency (WUE). WUE is calculated using the following formula (Heshmat et al., 2013):

\[
WUE \ (g/L) = \frac{\text{Biomass yield}}{\text{Total water used}}
\]

Where, biomass yield and total water usage are calculated at 0.5 m² harvest area.

Plants Sampling

Plants were sampled after 110 to 115 days when the aerobic rice has matured and ripened. Harvesting and sampling were done on a 1 m x 0.5 m area (0.5 m²). Total plant biomass was weighed to obtain the overall fresh weight. Plant parts such as straw, leaf and grain were separated and the fresh weight was measured. Straw, leaf, and grain samples were oven dried at 70 to 80°C for three days after which they were weighed (oven dry weight) and finely milled for further analysis. Total N in straw, leaf and grain was extracted by Kjeldahl digestion followed by steam distillation and subsequently 15N content was analyzed using emission spectrometer (IAEA, 2001). Percentage of 15N abundance was transformed into atom percentage 15N excess by subtracting the natural abundance (0.3663 atom %N) from the percentage of N abundance of the sample.

Samples Analysis

The Kjeldahl method is a means of determining the N content of rice samples. The Kjeldahl method may be broken down into three main steps; (i) Digestion, where the decomposition of N in plants samples utilizing a concentrated acid solution. This is accomplished by boiling a homogeneous sample in concentrated sulphuric acid. The end result is an ammonium sulphate solution. (ii) Distillation, where adding an excess base to the acid digestion mixture to convert NH₄⁺ to NH₃, followed by boiling and condensation of the NH₃ gas in a receiving solution and, (iii) Titration to quantify the amount of ammonia in the receiving solution (IAEA, 2001).
Emission spectrometer is an analytical instrument that can determine $^{15}$N isotope in samples. Samples were concentrated in liquid forms. Approximately 25 µl of plant samples were put in a glass capsule and placed in an emission spectrometer syringe. The emission spectrometer is equipped with a data acquisition unit which stores the data and automatically converts the measured to the true value.

NUE be calculated using the formula below (IAEA, 2001).

$$\% \text{ NUE} = \frac{\text{Fertilizer Nitrogen yield (kg/ha)}}{\text{Rate of Nitrogen application (kg/ha)}} \times 100$$

For $^{13}$C discrimination analysis, the dried leaf samples were homogenized by grinding. Sample of leaf was analyzed for $^{13}$C content by using Isotope Ratio Mass Spectrometer (IRMS). $\delta^{13}$C content was expressed in $\delta^{13}$C ‰ units using the International Pee Dee Belemnite Standard as shown below (Dercon et al., 2006):

$$\delta^{13}C (\text{‰}) = [(R_{\text{sample}})/(R_{\text{standard}} - 1)] \times 1000$$

Where, R is the ratio of $^{13}$C/$^{12}$C.

$\delta^{13}$C content of the plant material is related to $^{13}$C isotope discrimination ($\Delta^{13}$C) by the following equation:

$$\Delta^{13}C (\text{‰}) = [(\delta a - \delta p)/(1 + \delta p)] \times 100$$

Where $\delta a$ is the $\delta^{13}$C value of air (-8 ‰) and $\delta p$ is the measured value of the leaf samples.

**Statistical Analysis**

Analysis of variance (ANOVA) for variables that was used for testing treatments differences (water and N treatments) and independent T-Test to compare mean between two varieties was carried out. Correlation analysis was used to evaluate the relationship between N rates and grain yield. General linear model was used to testing interaction between treatments.
RESULTS AND DISCUSSION

Table 1: Analysis of variance effect of the treatments on the yield components, NUE, Δ^{13}C and WUE

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Level of significance at 5% level of probability</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Plant Height (cm)</td>
</tr>
<tr>
<td>Nitrogen Rates (N)</td>
<td></td>
</tr>
<tr>
<td>0 N</td>
<td>99.94a</td>
</tr>
<tr>
<td>60 N</td>
<td>112.57b</td>
</tr>
<tr>
<td>120 N</td>
<td>118.44b</td>
</tr>
<tr>
<td>P value</td>
<td>0.0001</td>
</tr>
<tr>
<td>Water Potentials (W)</td>
<td></td>
</tr>
<tr>
<td>ST</td>
<td>125.59c</td>
</tr>
<tr>
<td>FC</td>
<td>109.86b</td>
</tr>
<tr>
<td>SS</td>
<td>95.51a</td>
</tr>
<tr>
<td>P value</td>
<td>0.0001</td>
</tr>
<tr>
<td>Cultivars (C)</td>
<td></td>
</tr>
<tr>
<td>NMR151</td>
<td>110.17a</td>
</tr>
<tr>
<td>NMR152</td>
<td>110.47a</td>
</tr>
<tr>
<td>P value</td>
<td>0.899</td>
</tr>
<tr>
<td>Interactions</td>
<td></td>
</tr>
<tr>
<td>N x W</td>
<td>0.373</td>
</tr>
<tr>
<td>W x C</td>
<td>0.909</td>
</tr>
<tr>
<td>N x C</td>
<td>0.910</td>
</tr>
<tr>
<td>N x W x C</td>
<td>0.945</td>
</tr>
</tbody>
</table>

Means followed by the same letters in each column are not significantly different at 5% level of probability.

Yield Components

The experimental results for analysis of variance for yield components such as plant height, number of tillers, grain yield and weight of 1000 grains are summarized in Table 1. The results have shows, N rates and water potentials gave a significant effect on plant height, grain yield and 1000 grains weight for both cultivars. However, for the number of tillers only N rates had a significant effect on them but not water potentials. It may be due to the water potential treatments were applied to rice plants after tillering. If the water potential treatments are given before or during the formation of the tiller, water potential factors may influence the number of tillers (Bouman and Tuong, 2001). Plant height, number of tillers, grain yield, and 1000 grain weight at high N fertilizer applied (120 kg N/ha) showed the highest mean value compared to moderate and non-N fertilizer (60 kg N/ha and 0
kg N/ha. Jacqueline et al. (2008) also obtained a similar result, where N fertilizer rates had a significant effect on *Japonica* and *Indica* rice varieties and N fertilizer application rate at 120 kg N/ha gave the highest yield compared to the lower. However, N fertilizer applied at 60N and 120N showed no significant difference on plant height.

Plant height, grain yield and 1000 grain weight at lowest water potential (SS), resulted in the lowest mean value, as compared to ST and FC. However, although the grain yield mean at SS was the lowest but there was no significant difference with grain yield at FC. At ST, it was clearly shown that the grain yield was the highest and was significantly different from FC and SS. The study also showed there was no significant difference between NMR151 with NMR152 cultivars in relation to all yield components viz. plant height, number of tillers, grain yield, and 1000 grains weight. From this table, the interaction effect shows that all interaction in this study did not have significant effect on all yield components.

**Nitrogen Use Efficiency (NUE)**

The results of analysis of variance for NUE are summarized in Table 1. For the N treatments, the results show that at rate 0N there was no NUE value (NUE was not determined) because no N fertilizer applied to plants. At rates 60N and 120N, there was no significant difference between NUE values. Although there was no significant difference on NUE between N rates 60N and 120N, the NUE at rate 60N was found higher than rate 120N. This finding is similar to Mandana et al. (2011) who reported that increasing N application rates up to more than 60 kg N/ha caused NUE to decrease. Endris and Alemayehu (2014) also found the higher NUE in their study was not from the highest rate of N application to rice plants. The result also shows the water potentials had a significant effect on NUE. NUE at FC was higher than those of ST and SS. However, NUE at ST and SS water potentials were not significantly different from each other. These findings show that NUE of mutant line rice decreased at high (ST) and stress water conditions (SS). This result is similar to Nayak et al. (2015), who reported that water stress caused reduction of NUE, and Rahaman and Sinha (2013) who reported that NUE of rice was low under continuous flood condition.

These results indicated that the use of absorbed N from the soil by mutant line rice at the N rate of 60 kg N/ha under FC level was more efficient than others. High N fertilizer application up to 120 kg N/ha may increase the rice yields, but it also causes more N loss which reduces its effectiveness. These results also proved that changes in water level in the soil can influence the intake of N by plants. At ST and SS water potentials, the NUE was slightly lower than FC. Stagnant water conditions at ST level may have caused some N fertilizer loss through leaching and water runoff. Stress water condition like SS may disturb the vegetative growth of the plants that have caused the plant unable to absorb N in the soil efficiently. Dry soil conditions under water stress also may have disrupted the movement of plant roots to absorb N and may increase soil temperatures. The increase of soil temperature was reported to cause N loss through ammonia volatilization process and warm soil water cannot hold as much ammonia gas (Jones et al., 2013).

There was significant difference on NUE between NMR151and NMR152. NMR152 cultivar was found to have a higher NUE than NMR151 under different N rates and water potentials. Although there is no reference about these cultivars, Jacqueline et al. (2008) have reported in her study that different rice cultivars also have significant differences on NUE. Further research needs to be done on both cultivars to confirm the findings. The interaction effect shows all interaction tested in this study did not have a significant effect on NUE.
Carbon Isotope Discrimination ($\Delta^{13}C$)

Leaf $\Delta^{13}C$ values at Figure 1 ranged from 21.17 ‰ to 22.33 ‰ for NMR151 and from 20.97 ‰ to 22.25% for NMR152. Table 1 and Figure 1 shows a clear and significant water potentials effect on $\Delta^{13}C$ but N rates did not have a significant effect on $\Delta^{13}C$. Although N rates did not have a significant effect on $\Delta^{13}C$ in mutant line rice, the discrimination mean values at 0N nitrogen rate is found higher than 60N and 120N. $\Delta^{13}C$ in mutant line rice also decrease under stress water condition (SS) as compared to ST and FC conditions. These findings are in conformity with Shangguan et al. (2000) who reported the $\Delta^{13}C$ in another C₃ plant (wheat) decreased with increasing water stress and decreasing N application rates. These findings are in opposite of C₄ plants (maize) where the $\Delta^{13}C$ decreased with increasing water stress and increasing N rates application (Dercon et al., 2006).

![Figure 1: The influence of N rates on $\Delta^{13}C$ of mutant line rice under different water potentials. Error bars denote standard errors](image)

For both cultivars NMR151 and NMR152, $\Delta^{13}C$ was found not to have a significant difference from each other. For the interaction effects, only the interaction between N rates and water potentials had a significant effect on $\Delta^{13}C$. The other interactions were found not to have significant effects.

Water Use Efficiency (WUE)

Results in Table 1 show that both N rates and water potentials treatments clearly had significant effects on WUE. WUE increased with increasing nitrogen rates and increasing water stress. These findings are similar to that of Mandal et al. (2005), who reported WUE under low irrigation on rice was higher as compared to high irrigation. Zhang and Oweis (1999) has reported the use of water increased significantly with an increase of water supply at all rates of N application and WUE increased at low irrigation levels in comparison to high irrigation levels. These findings are in conformity with the Farquhar and Richards’s theory where WUE increased with increasing of N supply and with increasing water stress (Farquhar and Richards, 1984).
The Relationship between $\Delta^{13}C$ and WUE

Figure 2: The relationship between $\Delta^{13}C$ and WUE of mutant line rice under different water potentials and N rates

Figure 2 above shows a relationship between $\Delta^{13}C$ and WUE of the NMR151 and NMR152 mutant line rice under different N rates and water potentials. The result shows low but significant negative correlation between $\Delta^{13}C$ and WUE of the rice ($R = 0.487; P < 0.0001$). This relationship is in conformity to the report by Shangguan et al. (2000) on wheat ($C_3$ plant). This relationship also conforms to the theories of Farquhar and Richards (1984) where, high WUE has caused the ratio of intercellular carbon ($Ci$) on the concentration of carbon dioxide external ($Ca$) to reduces and causes the reduction of $\Delta^{13}C$ in plant.

CONCLUSIONS

As a conclusion, N rates and water potentials can affect the yield components such as plant height, grain yield, and 1000 grain weight of both mutant line rice cultivars NMR151 and NMR152. N rates also can affect number of tillers but not water potentials. Increasing N rates increased the yield but in some water condition, nitrogen use efficiency (NUE) was low despite high fertilizer rates. In this study, NUE was optimum at 60 kg N/ha under field capacity. Change in soil water potential from flood to aerobic condition was proven to improve the efficiency of N fertilizer uptake. However, when the soil is too dry, it can also reduce NUE. Avoiding over-fertilization is the utmost means to match a high use efficiency and economic return of N fertilizer with limited environmental risks from N loss. In this study, water potentials clearly affected $^{13}C$ isotope discrimination ($\Delta^{13}C$) and water use efficiency (WUE) and N rates only affect the WUE. Increasing N rate and water stress caused WUE to increase. All changes in $\Delta^{13}C$ values in both mutants line, whether resulting from changes in N supply or water potentials, were related to water deficits effects. WUE was clearly correlated with $\Delta^{13}C$ values in a way as it has been indicated in earlier reports. In addition, varying N rates is not causing any major complications with regard to the
relationship. For cultivar comparison, both NMR151 and NMR152 have similar results regarding plant height, number of tillers, grain yield, 1000 grain weight, Δ\(^{13}\)C, and WUE exception of NUE.

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