Effect of Fertilizer and Fixed Nitrogen on the Water Use Efficiency of Genge (*Astragalus sinicus* L.)

Akio Sumi, Shinpei Sugata, Ikumi Yahiro and Mina Odawara

(Faculty of Agriculture, Kagoshima University, 1-21-24 Korimoto, Kagoshima, Kagoshima 890-0065, Japan)

Abstract: To compare the effect of fertilizer nitrogen (N) and fixed N on water use efficiency (WUE), we measured the cumulative evapotranspiration (\(\sum ET\)) and total dry matter mass (DM) of genge growing in plant growth chambers. When regulating NH\(_4\)-N application at 11 levels ranging from 0 – 315 mgN flask\(^{-1}\), N concentration of the genge rose from 0.7% to 3.9% as fertilizer N application was increased. Although both DM and \(\sum ET\) changed on a quadratic curve, their relationship approximated a single regression line, regardless of N concentration. The effects of rhizobial inoculation of genge samples along with NH\(_4\)-N application rates of 0, 10.5, 105, and 252 mgN flask\(^{-1}\) were also tested. The samples with application rates of 0 and 10.5 mgN flask\(^{-1}\), where both nodulation and fixed N application were confirmed, had lower WUE than un-inoculated samples. These results suggest that the impact on WUE is much greater in fixed N than in fertilizer N.

Key words: Biological nitrogen fixation, Dry matter production, Evapotranspiration, Fertilizer nitrogen, Fixed nitrogen, Genge, Water use efficiency.

The agricultural consumption of nitrogen (N) fertilizer increases annually because N is one of the essential elements for improving agricultural production. Large amounts of fossil fuels are consumed in N industrial fixation; therefore, biological nitrogen fixation (BNF) is being investigated as a means of producing environmentally friendly renewable N (Bohlool et al., 1992; Jensen and Hauggaard-Nielsen, 2003). However, the biological cost of BNF is high (Ryle et al., 1979; Raven, 1985) and a drop in water use efficiency (WUE, g biomass kg\(^{-1}\) water transpired) in nodulated plants has been detected (Martínez-Carrasco et al., 1998). We also observed that nodulated plants consume more water than non-nodulated plants to produce the same dry matter mass (DM), resulting in a drop in WUE, and supposed that the difference between them reflects the DM cost used in driving BNF (Sumi et al., 2005; Sumi et al., 2011; Sumi, 2013). However, the question still remains whether the variation in WUE between nodulated plants and non-nodulated plants with their differing N metabolism can be considered to be a direct cost of N fixation. We need to answer this question before we can develop a new method for evaluating the amount of N fixed.

Although much attention has been focused on the impact of fertilizer N supply on WUE, results are not necessarily consistent (Brueck, 2008). Past many researches show that an increase in N supply accompanies an increase in WUE (Heitholt, 1989; Pieterse et al., 1997; Brück et al., 2001; Kiütük et al., 2004; Brueck and Senbayram, 2009). However, WUE is lower in nodulated plants with a high concentration of internal N (Unkovich and Pate, 2000; Sumi et al., 2005). Although these findings clearly supports the notion that BNF drives down WUE, this does not directly equate the difference in WUE to N fixation costs because the impact of the difference in internal N concentration between nodulated plants and non-nodulated plants on WUE is not removed. On the other hand, van den Boogaard et al. (1995) and Hobbie and Colpaert (2004) report examples where an increase in N supply actually lowered WUE. In this case, the reduction in WUE observed in nodulated plants casts doubt on the association with BNF and suggests that it is driven by N excess. The difference in WUE between nodulated plants and non-nodulated plants can only be directly related to N fixation cost when WUE has no relationship with the internal N conditions of plants (Hubick, 1990; Górny and Garczyński, 2002; Hamaoka et al., 2013).

There is an optimum curvilinear relationship between
plant growth rates and N concentration in culture medium (Hozumi et al., 1960); plant growth rates are reduced under either conditions of N shortage or N excess. The aims of this research are (1) to clarify the influence of fertilizer N on WUE under a wide range of N conditions and (2) to show that symbiotic N fixation reduces WUE in nodulated plants.

Materials and Methods

In this experiment we used 45 × 100 ml (135 ml capacity) triangular flasks each with 60 g of culture medium containing 0, 4.2, 10.5, 21, 42, 63, 105, 168, 210, 252, or 315 mgN flask⁻¹ in the form of ammonium sulfate \((\text{NH}_4\text{)}_2\text{SO}_4\). Triangular flasks were selected to minimize the evaporative surface; therefore, reducing the proportion of water lost by evaporation. The culture medium (seed soil EX, Green-sangyo Co., Ltd.) contained 150 mg N L⁻¹, 900 mg P₂O₅ L⁻¹, and 150 mg K₂O L⁻¹ and was confirmed free of any rhizobium that could form a symbiotic relationship with the genge plants (Sumi, 2013). Of the 45 flasks, 6 contained 0, 10.5, 105, or 252 mgN, whereas 3 flasks were prepared for each of the other application rates. Half of the previously mentioned 6 flasks with the 4 different application rates were inoculated with 0.2 g of rhizobium (for genge, Tokachi Nokyoren), whereas the rest were not inoculated. On February 8, 2013, pre-germinated seeds were planted at a density of 3 seeds per flask. Along with 4 others flasks prepared separately to monitor evaporation, the 49 flasks were immediately placed inside a plant growth chamber (Biotron LH200, Nihon Medical and Chemical Instruments Co., Ltd.) set at 20°C and a light intensity of 150 μmol m⁻² s⁻¹ (24-h continuous lighting). Subsequently they were watered once or twice a day according to their stage of growth and, at the same time, the position of each flask was changed to provide a uniform growing environment for all flasks. The plants were harvested on March 20 and were rinsed thoroughly to remove soil particle. After being dried for 72 h at a temperature of 82°C the total plant weight was determined. For plants that had been inoculated with rhizobium, their root nodules were also weighed. Evapotranspiration (ET) and evaporation \((E_0)\) rates were calculated by weighing the flasks before and after watering. WUE was defined as the ratio of DM to cumulative transpiration \((\Sigma T)\). Because cumulative evapotranspiration \((\Sigma ET)\) when DM is assumed to be 0 was in accord with cumulative evaporation \((\Sigma E_0)\) as shown later, \(\Sigma T\) was calculated by deducting \(\Sigma E_0\) from \(\Sigma ET\).

After being weighed, each plant was pounded in a mortar and the N content was measured twice using the Pregl-Dumas Method (J-Science Macro Corder JM1000CN). Each average N concentration was then multiplied by the plant weight to calculate total N content. In addition, the difference between the total N content of each inoculated plant and the average N content of those not inoculated with rhizobium were taken as the amount of N fixed.

The independent effect of fertilizer N was analyzed by using all data of un-inoculated genge from 0 to 315 mgN flask⁻¹ conditions, and statistically significant differences between fertilizer N treatments were given by LSD \((P = 0.05)\). On the other hand, the effects of rhizobial inoculation were analyzed by using the data under 0, 10.5, 105 and 252 mgN flask⁻¹ conditions, and statistically significant differences between inoculated and un-inoculated treatments were determined by the \(t\)-test.

Results

1. The influence of rhizobial inoculation and N fertilization on plant N concentration and amount of accumulated N

Nodulation was not observed at all when the prescribed rhizobium was not inoculated. Although nodulation due to rhizobial inoculation was observed, nodule weight dropped as N fertilization increased and no nodulation was observed at the 252 mgN flask⁻¹ level (Fig. 1). N concentration in plant \((N%)\) dropped from 3.9% to 0.7% in un-inoculated plants as N fertilization decreased from 252 to 0 mgN flask⁻¹, but N% in inoculated plants was kept at 3 – 2.7% even in the 0 – 10.5 mgN flask⁻¹ range (Fig. 2A). These measurements correspond to that in non-inoculated plants at the 168 mgN flask⁻¹ level (Fig. 2A). Although the amount of accumulated N (total N) in inoculated plants showed almost a straight line increase from 6 to 80 mgN flask⁻¹ with N supply up to the 210 mgN flask⁻¹ level, it actually dropped together with a drop in growth rate as shown later. Furthermore, the total N in the 0 – 10.5 mgN flask⁻¹ range increased with nodulation by approximately 40 mgN flask⁻¹ over un-inoculated plants (Fig. 2B). These values basically correspond to that in un-
inoculated plants at the 105 mgN flask$^{-1}$ level.

2. The influence of rhizobial inoculation and N fertilization on the relationship between DM and ET

In the relationship between DM and N fertilization there was optimum N condition at which DM is maximized (approximately 150 mgN flask$^{-1}$), when the rhizobial inoculation was not performed. DM in the 0 and 315 mgN flask$^{-1}$ dropped to 0.99 g and 0.72 g flask$^{-1}$ at a mere 39% and 28% of the maximum value, respectively, with t-test. Regression line given in (B) is applied to un-inoculated genge. * indicates that means between inoculated and un-inoculated genge at the same N level are significantly different at 5% level with t-test. Regression curve is applied to un-inoculated genge. (A), $y = -5.74 \times 10^{-3} (x - 75.2) + 2.38$, $R^2 = 0.99$, $P < 0.001$. (B), $y = -0.0107 (x - 152.1)^2 + 500.4$, $R^2 = 0.90$, $P < 0.001$. 

Letter ‘a’, ‘b’, ‘c’, ‘d’, ‘e’, ‘f’, ‘g’, ‘h’, ‘i’, ‘j’ and ‘k’ indicate ‘0’, ‘4.2’, ‘10.5’, ‘21’, ‘42’, ‘63’, ‘105’, ‘168’, ‘210’, ‘252’ and ‘315’ mgN application treatments, respectively.

---

Fig. 2. Effects of N fertilization and rhizobial inoculation on the nitrogen concentration (% dry weight) (A) and total N accumulated into plants (mg flask$^{-1}$) (B). Each data point represents the mean of 3 flasks. Bars represent LSD ($P = 0.05$) and is applied to un-inoculated genge. * and ** indicate that means between inoculated and un-inoculated genge at the same N level are significantly different at 5 and 1% levels, respectively, with t-test. Regression line given in (B) is applied to un-inoculated genge. $y = 0.353 x + 5.21 \ (0 \leq x \leq 210)$, $R^2 = 0.99$, $P < 0.001$.

Fig. 3. Effects of N fertilization and rhizobial inoculation on total plant dry mass (DM) (A) and cumulative evapotranspiration ($\sum ET$) (B). Each data point represents the mean of 3 flasks. Bars represent LSD ($P = 0.05$) and is applied to un-inoculated genge. * indicates that means between inoculated and un-inoculated genge at the same N level are significantly different at 5% level with t-test. Regression curve is applied to un-inoculated genge. (A), $y = -5.9 \times 10^{-3} (x - 145.9)^2 + 2.40$, $R^2 = 0.86$, $P < 0.001$. (B), $y = -0.0107 (x - 152.1)^2 + 500.4$, $R^2 = 0.90$, $P < 0.001$.

Fig. 4. Relationship between cumulative evapotranspiration ($\sum ET$) and total plant dry mass (DM). Regression line is applied to un-inoculated genge. $y = 5.74 \times 10^{-3} (x - 75.2)$, $R^2 = 0.99$, $P < 0.001$. Letter ‘a’, ‘b’, ‘c’, ‘d’, ‘e’, ‘f’, ‘g’, ‘h’, ‘i’, ‘j’ and ‘k’ indicate ‘0’, ‘4.2’, ‘10.5’, ‘21’, ‘42’, ‘63’, ‘105’, ‘168’, ‘210’, ‘252’ and ‘315’ mgN application treatments, respectively.
regardless of N fertilization, the relationship between DM (Y) and \( \sum ET \) (x) was approximated using the following formula (Fig. 4).

\[
Y \approx a \times (x - x_0)
\]  

(1)

In the above equation, slope ‘a’ represents WUE and in this experiment a figure of 5.75 ± 0.15 g DM kg\(^{-1}\) water transpired (mean ± s.e.) was obtained. In addition, \( x_0 \) is \( \sum ET \) when DM is assumed to be 0, and in this experiment, was in accord with 75.23 ± 7.77 g flak\(^{-1}\) (mean ± s.e., \( n = 4 \)) of \( \sum E_0 \) from bare flask, regardless of N fertilization. Equation (1) shows that fertilizer N had little effect on WUE, and regardless of drastic changes in N %, WUE was considered as practically the constant. On the other hand, DM in the 0 and 10.5 mgN flak\(^{-1}\) range where fixed N was applied is distributed below the line shown in Equation (1), whereas it showed a tendency to approach the line shown in Equation (1) at the 105 mgN flak\(^{-1}\) level where the difference in N% between inoculated plants and un-inoculated plants was the smallest (Fig. 2A). These facts suggest that the supply of fixed N lowered WUE.

**Discussion**

Although there has been much research on the influence of N supply on WUE, from uniform results have not always been obtained as described by Brueck (2008). There are many reports associating improvement of WUE with N supply (Heitholt, 1989; Pieterse et al., 1997; Brück et al., 2001; Brueck and Senbayram, 2009; Kütük et al., 2004). However, in this experiment, the increase in supply of fertilizer N and the accompanying increase in N%, showed no relation to WUE, which remained static (Fig. 4). This supports the results reported by Hubick (1990), Górny and Garchwaciónski (2002), and Hamaoka et al. (2013). However, Brueck and Senbayram (2009), who conducted a quantitative analysis of the influence of N supply on tobacco WUE, reported that WUE and DM proportionally increase with increases in N supply. Although it is impossible to find any clear cause for this disparity, it is feasible that the handling of \( \sum E_0 \) could be involved. More specifically, if the division of \( \sum E_0 \) is inconclusive, the calculated WUE is lower than actual WUE. The greater the proportion of \( \sum ET \) account for by \( \sum E_0 \) that is, the smaller DM, the greater the disagreement between both WUEs. In fact, even in experiments where we tried to reduce \( \sum E_0 \), the DM/\( \sum ET \) ratio showed a significant positive correlation with DM (\( r = 0.615, P < 0.05 \)) and resulted in the N conditions that produced maximum WUE (data not shown) as acknowledged by Clausen (2002). Similarly, the results shown in Fig. 4 do not constitute proof that WUE had no relationship with N% and was constant throughout the experiment period. Daily evapotranspiration rate (ETR) peaked quickly when N supply was low, after which they dropped (data not shown). Although the short-period WUE may also drop in line with this type of physiological process (Heitholt, 1989; Pieterse et al., 1997; Brück et al., 2001; Kütük et al., 2004), we could not detect such a reduction in integrated WUE throughout the experiment period (Fig. 4). In addition, ETR was initially low in cases where excessive N was applied, with delayed occurrence of maximum ETR (data not shown). This can be interpreted as N absorption accompanying growth and lowering the N concentration in the culture medium, resulting in the alleviation of growth restrictions due to N excess (Hozumi et al., 1960); however, no reduction in integrated WUE was detected (Fig. 4). Furthermore, even at the 315 mgN flak\(^{-1}\) level where DM dramatically dropped due to N excess, measurements for DM and \( \sum ET \) were distributed along the line shown in Equation (1) (Fig. 4). This experiment could not clarify the influence of fertilizer N on WUE.

On the other hand, through rhizobial inoculation the accumulated N in the 0 and 10.5 mgN flak\(^{-1}\) range was increased by approximately 40 mgN flak\(^{-1}\), which must have resulted from the supply of fixed N. WUE of nodulated genge can be calculated by dividing DM by the difference between \( \sum ET \) and \( \sum E_0 \), as \( \sum E_0 \) is determined to be a physical phenomenon, even when rhizobial inoculation is conducted, the 75.23 g flak\(^{-1}\) of Equation (1) is still applied. By contrast, the influence of fertilizer N on WUE of non-nodulated genge was undetected, WUE of nodulated genge decreased as the amount of N fixed increased (Fig. 5). In summary, the drop in WUE due to nodulation was not related to changes in N%, but was closely related to the action of BNF. In the present study, we estimated the amount of N fixed by
focusing on the difference in WUE between non-nodulated and nodulated plants. The nodulated genge also tended to have lower N use efficiency (NUE) than non-nodulated genge (data not shown). NUE is highly reliant on plant internal N conditions (Hamaoka et al., 2013). The finding that WUE of non-nodulated plants is a comparatively stable characteristic, whereas WUE of nodulated plants fluctuates with the amount of N fixed is of importance.

**References**

Bohlool, B.B. et al. 1992. *Plant Soil* 141: 111.

Brück, H. et al. 2001. *Plant Biol.* 3: 326-334.

Brueck, H. 2008. *J. Plant Nutr. Soil Sci.* 171: 210-219.

Brueck, H. and Senbayram, M. 2009. *J. Plant Nutr. Soil Sci.* 172: 216-223.

Claussen, W. 2002. *Plant Soil* 247: 199-209.

Górny, A.G. and Garciański S. 2002. *J. Appl. Genet.* 43: 145-160.

Hamaoka, N. et al. 2013. *Plant Prod. Sci.* 16: 107-116.

Heitholt, J.J. 1989. *Agron. J.* 81: 464-469.

Hobbie, E.A. and Colpaert, J.V. 2004. *New Phytol.* 164: 515-525.

Hozumi, K. et al. 1960. *Physiol. Ecol.* 9: 57-60*.

Hubick, K.T. 1990. *Aust. J. Plant Physiol.* 17: 413-430.

Jensen E.S. and Hauggaard-Nielsen, H. 2003. *Plant Soil* 252: 177-186.

Kitük, C. et al. 2004. *Aust. J. Soil Res.* 42: 345-341.

Martínez-Carrasco, R. et al. 1998. *Plant Cell Environ.* 21: 531-534.

Pieterse, P.A. et al. 1997. *Trop. Grassl.* 31: 117-123.

Raven, J.A. 1985. *New Phytol.* 101: 25-77.

Ryle G.J.A. et al. 1979. *J. Exp. Bot.* 30: 145-153.

Sumi, A. et al. 2005. *Jpn. J. Crop Sci.* 74: 344-349*.

Sumi, A. et al. 2011. *Jpn. J. Crop Sci.* 80: 190-198*.

Sumi, A. 2013. *Jpn. J. Agric. Educ.* 44: 47-55*.

Unkovich, M.J. and Pate, J.S. 2000. *Field Crop. Res.* 65: 211-228.

van den Boogaard et al. H. 1995. *J. Exp. Bot.* 46: 1429-1438.

* In Japanese with English abstract.