Mycorrhizae Effects on N Uptake and Assimilation Estimated by ^{15}N Tracing in White Clover under Water-Stressed Conditions

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^{15}N 추적에 의한 화이트 클로버에서 마이코라이자 접종이 수분 스트레스 조건하에서 질소 흡수 및 동화의 평가

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요 약

가뭄 스트레스 조건에서 마이코라이자의 접종이 질소의 흡수와 동화에 미치는 영향을 구명하기 위해 마이코라이자를 접종한 처리구와 접종하지 않은 처리구에서 수분처리 7일간 총 ^{15}N, 흡수된 NO_3^-로부터 아미노산과 단백질로 합성된 ^{15}N 함량을 각각 분석하였다. 정상 관수구에서는 전 조사항목에서 마이코라이자 접종에 대한 유의적인 효과가 나타나지 않았다. 총 ^{15}N 함량은 가뭄 스트레스에 의해 마이코라이자 접종구 및 비접종구에서 각각 13.8%, 28.5% 감소하였다. ^{15}N-NO_3^- 함량은 비접종구에서 유의적으로 증가하였다. 아미노산으로 합성된 ^{15}N 함량은 마이코라이자 비접종구에 비해 접종구의 잎과 뿌리에서 각각 1.26 및 1.33배 증가하였다. 가뭄 스트레스 조건의 잎에서 단백질로 합성된 ^{15}N 함량은 접종구보다 비접종구에서 더 높은 비율로 감소하였다. 이러한 결과는 가뭄 스트레스하에서 감소되는 질소흡수, 아미노산 및 단백질 합성을 마이코라이자 접종에 의해 효율적으로 경감시킬 수 있음을 잘 보여준다.

(**Key words** : Arbuscular mycorrhizae (AM), Drought, N uptake and assimilation, ^{15}N labeling)

Ⅰ. INTRODUCTION

White clover (*Trifoliumrepens* L.), the most important forage legume, extensively grows in temperate climate zones of the world (Kessler and Nösberger, 1994). It is considered one of very useful forage resources because of its excellent forage quality and high animal-feed value. However, the plant is often susceptible to drought owing to its shallow root system and inability to effectively control transpiration (Brink and Pederson, 1998). Additionally, water stress is one of the most important constraints on the growth and persistence of white clover (Barbour et al., 1995). Thus, a prolong drought decrease white clover yield, resulting in a decline in productivity of the livestock.

It is well known that nitrate is the main

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nitrogen source for most of the higher plants (Ruiz and Azcón, 1996), and it is the predominant N-form in many agricultural soils (Marschner, 1995). While soil moisture affects the movement of nutrients, especially those with a low diffusing capability, such as NH_4^+ (Azcón et al., 1996), meanwhile drought decreased mineralization of organically bound nutrients (Bloem et al., 1992; Walworth, 1992), which may diminish nutrient availability at the root surface.

Mycorrhizae with well-developed hyphae, however, increase the root surface area and thus are expected to aid the plant in the uptake of poorly diffused nutrients which become much less mobile in water-limited soils (Azcónet al., 1996). In addition, promoting more efficient absorption around the root, mycorrhizal hyphae are able to increase water uptake from the soil, thus improving drought tolerance (Azcón et al., 1988; Davies et al., 1992). Moreover, arbuscular mycorrhizal (AM) colonization stimulated activities of key enzyme involved in N assimilation such as nitrate reductase (NR), glutamine synthetase (GS) and glutamate synthase (GoGAT) (Subramanian and Charest, 1998). Mychorrhizae induced metabolic modifications and improved nutritional status by AM colonization appears to enable the host plant to withstand drought-stressed conditions in which the mobility of NO_3^- is severely restricted (Azcón et al., 1996).

We thus hypothesized that the AM fungus might have an effect on N uptake and assimilation in white clover exposed to drought conditions. To investigate this, we quantified total N uptake and the ^{15}N amounts incorporated into amino acid, soluble proteins both in leaves and roots under well-watered and drought-stressed conditions.

Ⅱ. MATERIALS AND METHODS

1. Plant culture

Seeds of white clover (*Trifoliumrepens* L.) were surface-sterilized by a consecutive washing with Tween 20 and 80% ethanol (Kim et al. 1991). Three uniform seedlings per pot were transplanted after 15 days to 3 L pots containing a sterilized mixture of quartz sand and soil. The experimental soil had a pH of 7.6, 1.9% organic matter, 0.09% total N, 37 μg g^{-1} ($NaHCO_3$-extractable P) and 351 μg K g^{-1}. For the pots of mycorrhizal treatment, five grams of *Glomus intraradics* inoculum, containing hyphae, spores, mycorrhizal root fragments, and soil was placed directly below the seedling through plastic tube. Each pot was watered daily to full capacity with 200 ml of complete nutrient solution (Kim et al., 1991) in order to prevent mineral nutrient deficiencies. The seedlings were cultivated in a greenhouse with a day/night mean temperature of 27/18°C, and a relative humidity of 65/80%. In addition to natural light, supplemental light was by metal halide lamps which generated approximately 400 μM photons m^{-2} s^{-1} at the canopy height for 16 h per day.

2. Drought-stressed imposition and $^{15}NO_3^-$ labeling

Plants were regularly watered to field capacity until full vegetative stage (approximately 12 weeks after sowing). At this stage, water-deficit stress was imposed by decreasing amount of irrigation for 7 days. 200 mL and 20 mL of daily irrigation per pot were applied for natural irrigation and drought treatment respectively. Half of the daily irrigation volume for each

treatment was applied at 10:00 h and the remaining half at 16:00 h. Three porous plastic tubes (1.5 cm diameter and 8 cm height) were buried vertically to a depth of 5 cm (leaving 3 cm of each tube above the soil surface) in each pot, to directly deliver the applied water to the root zone. For ^{15}N feeding for the well-watered (control) treatment, 100 mL of ^{15}N solution (1 mM $K^{15}NO_3$ with 8.34 ^{15}N atom% excess) was administered evenly through three tubes placed in each pot at 10:00 h and 16:00 h, respectively. For the water-deficit treatment, 5 mL of ^{15}N solution, containing the same amount of N as supplied to the control pot (corresponding to 0.7 mg N pot^{-1} d^{-1}) with the same ^{15}N atom%, was applied as described for the control plants. The $^{15}NO_3^-$ feeding was done every day throughout the entire 7-d sampling period.

3. Measurements of sampling

The first sampling, day 0, was made just before the start of the water-stress treatment (the first $^{15}NO_3^-$ feeding) at 10:00 h. Additional sampling times were 1, 3, 5 and 7d after treatment. For chemical and isotope analysis, three plants in each pot were combined after the measurement of leaf water parameters. Harvested plants were separated into leaves and roots. All plant samples were immediately frozen in liquid nitrogen, freeze-dried, weighed, round and stored in vacuum desiccators for further analysis.

4. Chemical and isotope analysis

Soluble proteins were extracted twice from approximately 200 mg of finely ground freeze-dried sample with 100 mM sodium-phosphate buffer (pH 7.5) at 4℃. Proteins in the combined supernatant were precipitated with 80% (v/v) acetone and centrifuged at 10,000 g for 10min at 4℃. The pellet from this precipitation was dissolved with 0.5 mL of extraction buffer. For liquid samples fractionated, an appropriate sample volume (usually 0.1 mL) containing more than the minimum quantities (25 μg N) was dropped into a tin capsule that had been cooled with liquid nitrogen. The contents of tin capsules were then freeze-dried.

Freeze-dried powder samples (1~5 mg) were weighed (±10 μg) into a tin capsule for total N determination. N content and ^{15}N atom% of all fractions was determined by N single mode analysis on an ANCA-SL isotopic ratio mass spectrometer (PDZ-Europa, Crewe, UK). The obtained ^{15}N abundances were converted to relative specific activities (RSA, i.e. % of recently incorporated atoms relative to the total numbers of atoms in the sample) by eqn 1,

RSA = (^{15}N atom% in the nitrogenous compounds - natural ^{15}N atom%) / (^{15}N atom % of administered $^{15}NO_3^-$-natural ^{15}N atom%) × 100 (eqn 1)

The natural ^{15}N atom% was adopted from the ^{15}N atom% of non-^{15}N-fed plants. The amounts of newly absorbed N in the nitrogenous compounds (NNC) were calculated by eqn 2,

NNC = (RSA × N content measured in the nitrogenous compounds) / 100 (eqn 2)

5. Statistical Analysis

A completely-randomised design was used with three replicates for AM and non-AM plants under two water levels and five sampling dates. An individual pot containing three plants represented a replicate. Duncan's multiple range tests were used to compare the means of

separate replicates. Unless otherwise stated, conclusions are based on differences between the means, with the significant level at $p = 0.05$.

Ⅲ. RESULTS

1. Nitrogen uptake

N uptake was estimated by the sum of ^{15}N amount found in leaves and roots. Under well-watered (WW) conditions, total ^{15}N was not significantly different both in roots and leaves, regardless AM symbiosis. By contrast, this amount was significantly ($p<0.05$) lower in leaves of plants exposed to drought stress throughout the experiment period, regardless of AM colonization (Fig. 1). Drought stress (DS) rapidly decreased total ^{15}N amount from the first day of treatment both in AM and non-AM plants. Nevertheless, the rate of decrease in whole plant caused by drought was much less in AM plants (-47% at day 1) than non-AM plants (-77% at day 1). After 7 days of drought treatment, total ^{15}N amount in AM plants was 15% higher than that of non-AM ones.

2. The amount of ^{15}N incorporated into NO_3^-, amino acids and soluble proteins

To determine the amount of non-reduced N, the ^{15}N amount in NO_3^- was estimated (Table 1). Under well-watered conditions, there was no significant difference both in leaves and roots throughout the experimental period, regardless of AM colonization. Under drought-stressed condition, AM symbiosis effect on ^{15}N-NO_3^- concentration was apparent from day 5 in leaves and only at day 7 in roots, with a higher in non-AM relative to AM plants.

To determine N amount incorporated into amino acids, ^{15}N amount in amino acids fraction derived from the newly absorbed N was analyzed

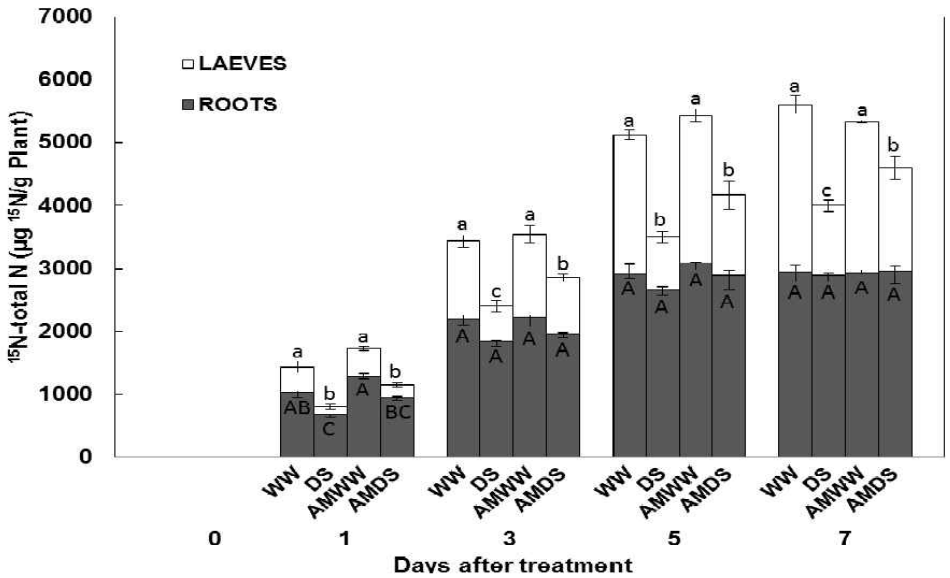

Fig. 1. Total ^{15}N amount distributed inleaves and roots under well-watered (WW) and drought-stressed (DS) conditions for 7-day treatment. The values are means ± SE of triplicates, followed by different capital for roots or small letters for leaves are significantly different at $p<0.05$ according to Duncan's multiple range tests.

Table 1. Changes in the amount of ^{15}N in NO_3^- fraction in leaves and roots of mycorrhizal colonized (AM) and non-colonized (non-AM) white clover under well-watered (WW) and drought-stressed (DS) conditions

Parameters / Treatment		Days after treatment			
		1	3	5	7
Leaves (μg ^{15}N/g DM)					
WW	AM	12.89^{ab}	27.42^{a}	30.94^{a}	57.42^{ab}
	Non-AM	15.50^{a}	23.71^{ab}	32.96^{a}	61.72^{a}
DS	AM	11.52^{ab}	11.58^{c}	22.73^{b}	46.44^{c}
	Non-AM	7.64^{b}	13.19^{bc}	31.23^{a}	53.29^{b}
Roots (μg ^{15}N/g DM)					
WW	AM	241.41^{a}	463.35^{b}	582.83^{c}	665.69^{c}
	Non-AM	277.60^{a}	521.58^{b}	613.80^{bc}	701.18^{c}
DS	AM	236.67^{a}	496.92^{b}	721.34^{ab}	855.73^{b}
	Non-AM	266.85^{a}	608.84^{a}	824.82^{a}	938.58^{a}

Values are means of triplicate. Within the same column, means followed by the same letters are not significantly different at $p<0.05$ according to Duncan's multiple range tests.

Table 2. Changes in the amount of ^{15}N incorporated into amino acids from the newly absorbed N in leaves and roots of mycorrhizal colonized (AM) and non-colonized (non-AM) white clover under well-watered (WW) and drought-stressed (DS) conditions

Parameters / Treatment		Days after treatment			
		1	3	5	7
Leaves (μg ^{15}N/g DM)					
WW	AM	74.17^{ab}	170.06^{a}	244.88^{a}	339.00^{ab}
	Non-AM	74.00^{ab}	150.45^{a}	246.21^{a}	347.79^{ab}
DS	AM	71.92^{a}	181.06^{a}	251.24^{a}	403.63^{a}
	Non-AM	93.48^{b}	213.39^{a}	253.29^{a}	319.20^{b}
Roots (μg ^{15}N/g DM)					
WW	AM	14.39^{b}	20.79^{a}	31.50^{a}	43.75^{a}
	Non-AM	14.46^{b}	22.38^{a}	31.04^{a}	44.91^{a}
DS	AM	19.23^{b}	24.30^{a}	30.65^{a}	30.82^{b}
	Non-AM	27.20^{a}	28.88^{a}	24.96^{a}	23.26^{b}

Values are means of triplicate. Within the same column, means followed by the same letters are not significantly different at $p<0.05$ according to Duncan's multiple range tests.

(Table 2). For the well-watered plants, there was no significant difference in ^{15}N-amino acids concentration between AM and non-AM plants. Under drought-stressed condition, a significantly higher concentration was observed at the first day in non-AM plants, this intrinsic increase was not continued afterwards. At the final day of sampling, ^{15}N amount in amino acids in AM plants increased 1.26-fold and 1.33-fold, respectively, in leaves and roots compared to non-AM ones.

Drought sharply decreased ^{15}N amount in soluble proteins fraction both in leaves (from day 3) and roots (for the first 3 days), regardless of AM colonization, representing 24.5% and 52.8%, respectively, in AM and in non-AM plants at 7 days after drought (Table 3). AM symbiosis did

Table 3. Changes in the amount of ^{15}N incorporated into proteins from the newly absorbed in leaves and roots of mycorrhizal colonized (AM) and non-colonized (non-AM) white clover under well-watered (WW) and drought-stressed (DS) conditions

Parameters / Treatment		Days after treatment			
		1	3	5	7
Leaves (μg ^{15}N/g DM)					
WW	AM	8.52[a]	81.08[a]	115.54[a]	148.44[a]
	Non-AM	10.21[a]	81.97[a]	112.92[a]	145.32[a]
DS	AM	5.34[a]	40.34[b]	75.82[b]	112.05[b]
	Non-AM	4.52[a]	32.47[b]	60.05[b]	68.52[c]
Roots (μg ^{15}N/g DM)					
WW	AM	28.04[a]	153.19[a]	276.79[a]	355.70[a]
	Non-AM	30.65[a]	158.04[a]	268.84[a]	327.51[ab]
DS	AM	12.56[b]	125.21[b]	229.97[ab]	311.85[ab]
	Non-AM	10.31[b]	111.37[b]	199.25[b]	266.68[b]

Values are means of triplicate. Within the same column, means followed by the same letters are not significantly different at $p<0.05$ according to Duncan's multiple range tests.

not have significant influence on the ^{15}N amount in proteins both in leaves and roots. However, under drought-stressed condition, the decrease in ^{15}N amount in proteins both in leaves and roots. However, under drought-stessed condition, the decrease in ^{15}N amount in proteins was much alleviated by AM colonization, so that the ^{15}N amount in drought-stressed AM leaves was 1.63-fold higher than that of non-AM ones.

Ⅳ. DISCUSSION

Total ^{15}N amount was significantly decreased ($p<0.05$) by drought stress (Fig. 1), indicating that drought stress restricts the nitrogen acquisition from soil, it could be suggested that the restriction N uptake results in the signi-cantly decrease of the ^{15}N-total N amount in leaves. Previous studies have showed that a reduction of N amount is a common observation under stressed conditions (Dubay and Pessarakli, 1995), which can be interpreted as a reduction in N availability and the limitation of both N acquisition and nitrate reductase activity under stressed condition (Aslam and Huffaker, 1984; Rao and Gnanam, 1990). By contrast, AM white clover has a higher total ^{15}N amount under water stress conditions (Fig. 1), indicating that hyphal network could be a significant factor in N uptake and as such play a key role in plant N status. It has been established that AM hyphae help the plant to take up soil nutrient by increasing the roots surface area (Barea, 1991), under especially water-limited condition. Tobar et al. (1994) provided evidence of hyphal transport of N and estimated four times higher mass flow and NO_3^- diffusion in AM plants than in non-AM plants by ^{15}N-NO_3^- tracing method. These results are well consistent with our ^{15}N uptake data, suggesting that AM symbiosis may be an effective potent to improve the acquisition of mineral nutrients in dry soils.

Under drought-stressed condition, ^{15}N-NO_3^- concentration significantly higher in non-AM than in AM plants after 5 days of treatment (Table 1). This may be due to the inhibited assimila-

tion of NO_3^- newly absorbed into amino acids and proteins. Kim et al. (2004) and Lee et al. (2005) have reported that drought stress significantly decreased *de novo* synthesis of amino acids and proteins at the terminal drought period, resulting in the accumulation NO_3^- in roots, especially. In addition, the earlier research showed that drought stress restricted nitrate translocation from roots to leaves (Lee et al., 2007), as a consequence of nitrate accumulation in roots.

In this study, a significantly higher ^{15}N amount in amino acids fraction was observed at the first day in both leaves and roots of non-AM plants (Table 2). This intrinsic increase may be a transient response for the drought stress. Lee et al. (2005) have also found an increase in amino acids pool in the early period of drought. They suggested that is associated with the increased ammonia concentration, which in turn arise in response to decrease in *de novo* protein synthesis. However, the amount of ^{15}N-amino acids in leaves reversely higher in AM plants than in non-AM ones at the final drought treatment (day 7). This indicates that AM colonization effectively mitigates the decline of *de novo* amino acids synthesis caused by drought. In the present study, the amount of ^{15}N-soluble proteins was significantly decreased by drought stress regardless of AM colonization. AM symbiosis effects was not appeared in well-watered conditions, meanwhile the amount of ^{15}N-soluble proteins was significantly higher in AM leaves than in non-AM ones at 7 day of drought treatment (Table 3). The results are consistent with Lee et al. (2005) who observed a marked reduction in the *de novo* protein synthesis from the newly absorbed N in drought-stressed white clover plants, particularly in leaves.

Taken together, AM symbiosis may be an effective strategy for drought tolerance by alleviating the decrease in N uptake and N assimilation caused by drought, which is a common stress factor resulting in a limitation of the synthesis of amino acids and proteins.

Ⅴ. ABSTRACT

To investigate the effects of arbuscular mycorrhizal (AM) symbiosis on N uptake and its assimilation under drought-stressed conditions in white clover, total ^{15}N amount and ^{15}N amount incorporated into NO_3^-, amino acids and soluble proteins were quantified by ^{15}N tracing during 7 days of water treatment. Under well-watered conditions, there were no significant effects of AM symbiosis on all parameters analyzed in this study. Drought stress decreased total ^{15}N amount both in AM and non-AM plants, with a lower rate in AM plants (-13.8%) relative to non-AM plants (-28.5%) at day 7. Drought significantly increased ^{15}N-NO_3^- amount in non-AM plants. The amount of ^{15}N-amino acids was 1.26-fold and 1.33-fold higher, respectively, in leaves and roots of AM plants compared to those of non-AM ones. Drought decreased the amount of ^{15}N-soluble proteins in leaves at day 7, with a higher rate in non-AM plants than in AM ones. These results clearly indicate that AM colonization effectively alleviating the decrease in N uptake, amino acids and proteins synthesis caused by drought stress.

Ⅵ. ACKNOWLEDGEMENT

"This work was supported by the Korea Research Foundation Grant funded by the Korean Government (MOEHRD)" (KRF-2007-313-F00056)

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(접수일: 2011년 6월 10일, 수정일1차: 2011년6월23일, 수정일2차: 2011년 7월15일, 게재확정일: 2011년 8월 5일)