# Estimation of nitrogen fixation rate of soybean (*Glycine max* (L.) Merr.) by micro-scale relative ureide analysis using root bleeding xylem sap and apoplast fluid in stem.

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**Summary**

Relative ureide method is a simple and reliable method for estimating the percentage of nitrogen depending on nitrogen fixation in field grown soybean plants. Generally, the concentrations of ureides, amides and nitrate in root bleeding xylem sap from a cut lower main stem are determined colorimetrically using an optical spectrometer. We developed a micro-scale analysis of these components in xylem sap using 2.5 μL of xylem sap for each analysis instead of 50 μL for the standard assays. Each reaction was carried out in a 1.5 mL Eppendorf centrifuge tube, and the 200 μL of reaction mixture was put into a well of a 96 wells-microplate, and measured by a microplate reader. The duplicated analysis of the standard solution gave accurate calibration for each assay. We can analyze about 200 samples in one day by the micro-scale analysis. In addition, we tried to compare the stem fluid collected by centrifuge of the lower stem segments, because root bleeding sap cannot be collected under dry conditions or at late stages such as R7 stage. Stem fluid was always collected by a portable centrifuge at 2,200 x G for 10 seconds, and the volume increased from R1 to R7 stages. The concentrations of nitrogen components and the relative ureide percent in root bleeding xylem xap and stem fluid were similar at R7, but different at R1 and R5 stages. The relative ureide percentages from stem fluid were about 50% at R1 and R5, but those from xylem sap were about 80-90%. Therefore, the stem fluid may be used for relative ureide percent only at R7 stage, when xylem sap is sometimes difficult to obtain.

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**Key words** : Relative ureide method, Soybean, Microplate, Xylem sap, Stem fluid

## Introduction

Soybean plants require a large amount of nitrogen compared with cereal crops, such as rice or wheat, and the N is derived from three sources; N derived from atmospheric $N_2$ (Ndfa) by symbiotic nitrogen fixation, N derived from soil (Ndfs) and N derived from fertilizer (Ndff). To improve the N fertilization or cultivation methods, it is important to evaluate the percentage proportion of the N origins.

A relative ureide method is a simple and reliable method for estimating the percentage of nitrogen depending on nitrogen fixation (%Ndfa) in a field grown soybean (Herridge et al. 1990, Takahashi et al. 1992, 1993). Recently papers have been published using a relative ureide method for estimating %Ndfa (Nohara et al. 2005, Nagumo et al. 2010, Takakai et al. 2010, Murata et al. 2012). This method is based on the fact that Ishizuka et al. (Kushizaki et al. 1964, Ishizuka 1970) found that nodulating soybean accumulated a high concentration of ureides (allantoin and allantoic acid) in the stem. Then it was found that the principal transport form of N fixed by the nodules is mainly ureides (Matsumoto et al. 1977, Ohyama and Kumazawa 1978), but major forms of N from roots are nitrate and amides (Ohyama and Kumazawa 1979, Ohyama et al. 2009). MacClure and Israel (1979) analyzed transport of nitrogen in the xylem sap of soybean plants, and proposed the percent ureide-N in xylem sap may be used as an indicator of the relative contribution of $N_2$ fixation to the total input of plant N. Herridge and Peoples (1990) compared the ureide assay and $^{15}N$ method, and they proposed the calibration equations. They calculated the relative abundance of ureide-N in root bleeding sap, and vacuum-extracted sap by (100 x ureide-N/(ureide-N + $\alpha$-amino-N + nitrate-N), and stem hot water extracts by (100 x ureide-N/(ureide-N + nitrate-N). We proposed the eqation as 100 x ureide-N/(ureide-N + amide-N + nitrate-N), where amide-N is "2 x $\alpha$-amino-N", because major form of amino acids and amides is asparagine (McClure and Israel, 1979, Herridge 1984, Ohyama et al. 1989). When plants are periodically harvested (eg. R1, R5 and R7 stages), and the nitrogen fixation activity and nitrate absorption rate could be estimated from the relative ureide percent and total N increase in the shoots (Takahashi et al. 1992, 1993, Ohyama 1990, Tewari et al. 2004, 2011, Ohyama and Toan 2006).

For relative ureide analysis, the concentrations of

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ureides, amides and nitrate in xylem sap bleeding from the cut lower main stem are determined colorimetrically using an optical spectrometer. Sometimes it is not successful for collecting xylem sap, especially when soil is dry or during late growth stage at R7. Herridge and Peoples (1990) used a vacuum extracted stem exudate from lower part of the main stem of the cut shoot or hot-water extraction of the stems.

By standard analysis of ureides, amides, and nitrate need 50 μL of sample solution each, so at least 150 μL of xylem sap is necessary for estimating relative ureide percent. In addition, it was time-consuming to analyze each component by using test tubes. Usually we can analyze only about 20-50 samples per a day. We developed a micro-scale analysis of these components in xylem sap using 2.5 μL of xylem sap

**Fig 1.** Photograph of xylem sap collection by inserting a tube into a cut stem of soybean plant grown in the field. Bark was removed before fitting the tube.

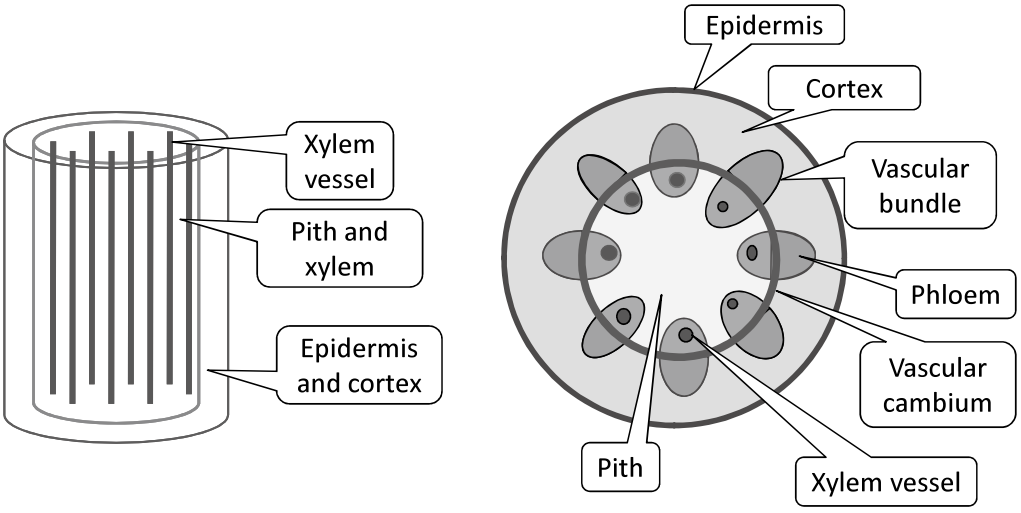


**Fig 2.** Structure of the stem.

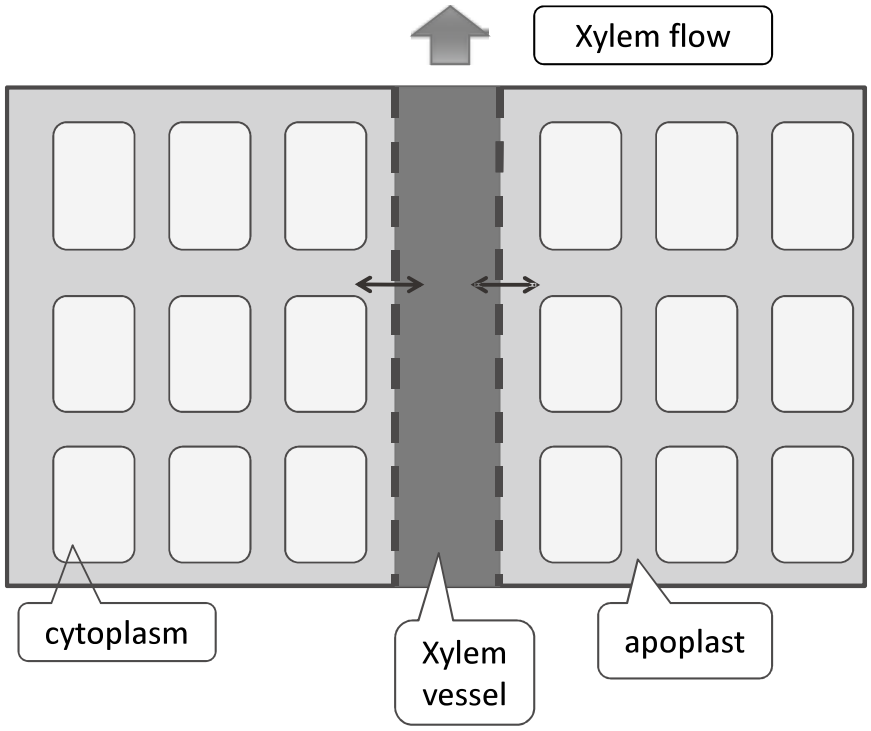


**Fig 3.** Water transport in a xylem vessel and surrounding stem tissue.

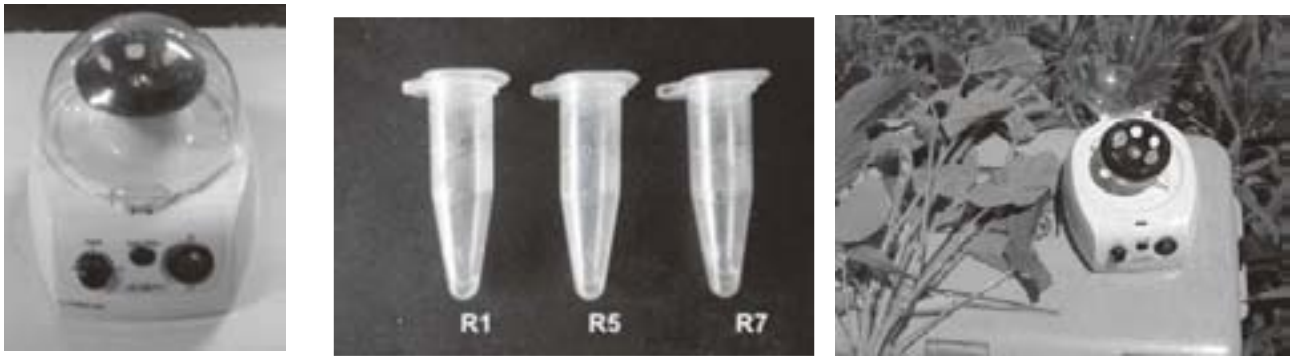


**Fig 4.** Photographs of stem fluid collection.
A: Portable centrifuge driven by 1.5 V batteries.
B: Stem fluid collected at R1, R5 and R7.
C: Photograph of stem fluid collection in the field.

instead of 50 μL for standard assay. The colorimetric reaction was carried out in a 1.5 mL Eppendorf centrifuge tube, and the 200 μL of reaction mixture was put into the well of a 96 wells-microplate, and the optical absorbance can be measured by a microplate reader. The analysis using duplicated standard solution gave accurate calibration for each assay.

The root bleeding xylem sap was collected as shown in Figure 1. The lower part of the main stem just below the node with cotyledons was cut by a pair of pruning shears. The tigon tube was inserted into the stem woody part. Xylem sap started to exude at several minutes after cutting by a root pressure. The structure of the stem was shown in Figure 2. In the bark of the stem there are epidermis and cortex including vascular bundles with phloem. In the central woody part of the stem there are xylem vessels and pith. Xylem sap comes up through xylem vessels in the stem from root xylem vessels via transpiration stream and root pressure. As shown in Figure 3, xylem vessel is not closed pipe, but they have pits on the wall and water and solutes move to the apoplast from xylem vessels and also liquid in apoplast come back to xylem vessels (Ohya et al. 2008).

We tried to collect stem fluid by centrifuge of the stems

at lower part, because root bleeding sap cannot be collected under dry conditions or at late stages such as R7 stage (Figure 4). Stem exudate was always collected by a portable centrifuge at 2,200 x G for 10 second.

**Materials and Methods**

***Micro-scale analysis of relative ureide method.***

Usually 50 μL of xylem sap was used for each analysis. However, the volume of xylem sap is sometimes very low and not enough for the relative ureide analysis. So we scale down the volume of each analysis to 2.5 μL for each analysis. We use a 1.5 mL Eppendorf centrifuge tube instead of a large glass tube. The colorimetric analysis of ureide-N, amide-N, and nitrate-N are essentially the same as the previous reports (Takahashi et al. 1992, 1993, Ohyama 1990), except for using 1/20 volume of sample and all reagents added. 5 mM solution of amides solution (2.5 mM glutamine + 2.5 mM asparagine), 5 mM of sodium nitrate solution and 5 mM allantoin are used for standard solution for calibration.

The final volume is 527 μL, 473 μL, and 262 μL for ureide-N, amide-N, and nitrate-N analysis. 200 μL of the reaction solution was put into a flat-bottom microplate (96 wells, 8 x 12), and the absorbance was measured at 520 nm, 570 nm, and 410 nm for ureide-N, amide-N, and nitrate-N analysis by microplate reader (Multiskan Spectrum, Thermo Electron, Vantaa, Finland).

The standard-scale analysis was carried out (Takahashi et al. 1992, 1993, Ohyama 1990, Ohyama and Toan 2006), and the absorbance was determined by a spectrophotometer (UV mini-1240, Shimadzu, Kyoto, Japan) using a standard-size cuvette (optical path 1cm).

***Analytical procedures for ureide concentration.***

Ureide concentration was determined by the method of Young and Conway (1942). 2.5 μL of sample solution or standard solution was taken into a 1.5 mL Eppendorf tube, and 300 μL of 83 mM NaOH (332 mg of NaOH was dissolved in 100 mL of water) was mixed, and heated at 100 ℃ for 8 min in an aluminum block heater (Dry Thermo Unit DTU-2C, TAITEC, Koshigaya, Japan). After cooling the solution about 10 min, 100 μL of ice-cold phenylhydrazinium cloride solution (165 mg of phenylhydrazinium cloride was dissolved in 50 mL of water and mixed with 50 mL of 0.65 M HCl (2.71 mL of concentrated HCl (12 M $L^{-1}$) was filled up to 50 mL with water). The tube was heated in aluminum block heater at 100 ℃ for 2 min, and immediately cooled in iced water for 15 min. Then 125 μL of cold potassium ferricyanide solution (334 mg of potassium ferricyanide was dissolved in 20 mL of water and mixed with 80 mL of 10 M HCl (mix 83.3 mL of conc. HCl (12M $L^{-1}$) was filled up to 100 mL by water) was added and mixed well. After 30 min in ice-cold water, the absorption at 520 nm was measured by microplate reader. Standard solution was made by dissolving 79.1 mg of allantoin in 100 mL of water, which contains 5 mM allantoin (280 mgN $L^{-1}$). Diluted solution (0, 1, 2, 3, 4, 5 mM) was used for calibration.

***Analytical procedures for amide concentration.***

Amide concentration was determined by ninhydrin method (Herridge 1984). 2.5 μL of sample solution or standard solution was taken into a 1.5 mL Eppendorf tube, and 75 μL of citrate buffer (5.60 g of citrate and 2.13 g of NaOH was dissolved in 100 mL of water) was mixed. Afterwards, 60 μL of ninhydrin solution (958 mg of ninhydrin and 33.4 mg of ascorbate was dissolved in 3.2 mL of water and fill up to 100 mL with methoxyethanol (methylcellosolve)) was added. The lid of the tube was closed then heated in aluminum block heater (Dry Thermo Unit DTU-2C, TAITEC, Koshigaya, Japan) at 100 ℃ for 20 min. 300 μL of ethanol was added and cooled to room temperature for 10 min. 200 μL of the reaction solution was put into a microplate, ant the absorption at 570 nm was measured by a microplate reader. Standard solution was made by dissolving 66.1 mg of asparagine (or 70.1 mg of asparagine monohydrate) plus 73.1 mg of glutamine in 100 mL of water, which contains 5 mM asparagine + 5 mM glutamine (280 mgN $L^{-1}$). Diluted solution (0, 28, 56, 84, 112, 140 mgN $L^{-1}$) was used for calibration.

***Analytical procedures for nitrate concenetration.***

Nitrate-N concentration was determined by Cataldo's method (Cataldo et al. 1974). 2.5 μL of sample solution or standard solution was taken into a 1.5 mL Eppendorf tube, and 10 μL of salicylic acid-sulfate solution (500 mg of salicylic acid was dissolved in 10 mL of concentrated sulfuric acid) was mixed and kept for 20 min. Then, 250 μL of 2 M NaOH solution (8.00 g of NaOH was dissolved in 100 mL of water) was mixed and keep it for 20 min. 200 μL of the reaction solution was put into a microplate, ant the absorption at 410 nm was measured by microplate reader. Standard solution was made by dissolving 42.5 mg of $NaNO_3$ in 100 mL of water, which contains 5 mM nitrate (70 mgN $L^{-1}$). Diluted solution (0, 1, 2, 3, 4, 5 mM) was used for the calibration.

Figure 5 shows the procedures of micro-scale analysis. Eppendorf tubes are heated in aluminum block heater (Figure 5 A, B, C). The tubes are cooled in ice water for ureide analysis. (Figure 5 D). The reaction mixture was put into a microplate (Figure 5, E, F).

***Collection of root bleeding xylem sap and stem fluid.***

The main stem at the height of about 5 cm aboveground was cut by sharp pruning scissors, and a tigon tube with 5 cm long and 5-10 mm diameter was put on the stem. Xylem sap was collected about 1 hour.

In addition, the lower main stem segments from basal stem to 3$^{rd}$ node were cut into 2-3 cm long segments, and put into a 1.5 mL Eppendorf tube. The apoplast fluid was collected by centrifuged at 6,500 rpm (2,200 x G) for 10 second using a portable centrifuge (Figure 2, AcNoPlus, ERMA Inc., Tokyo, Japan). This centrifuge can be driven by 1.5 V batteries, so we can use in soybean fields (Figure 4). The clear fluid can be corrected at the bottom of the Eppendorf tube (Figure 4 B).

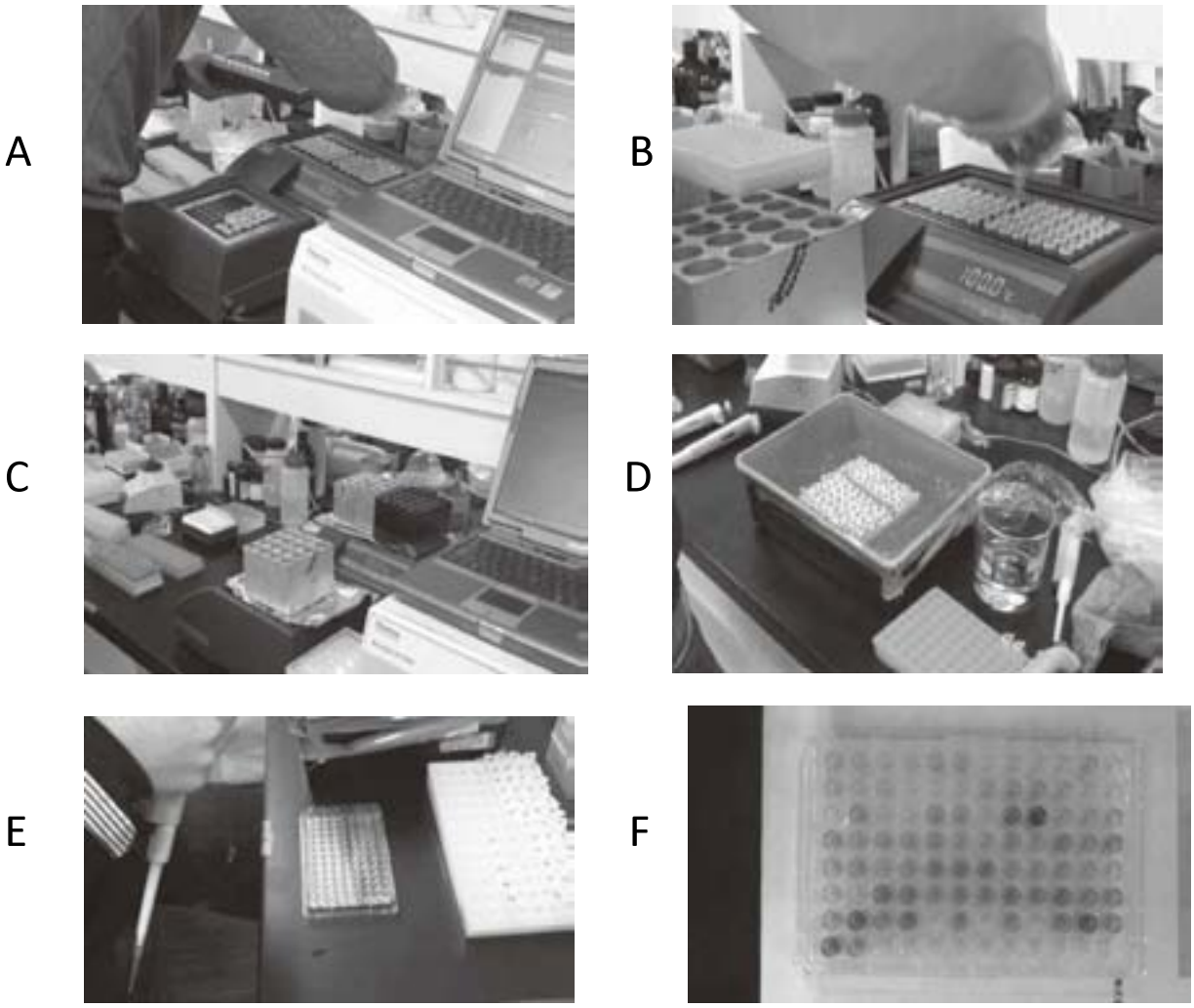


**Fig 5.** Procedures of micro-analysis for relative ureide abundance.
A and B: 1.5 mL Eppendorf tubes were used for all the analysis. Alminum block heater is used instead of boiling water.
C: Aluminum blocks are put on tubes while heating, not to open the lid.
D: Tubes are put in a cold box with iced water when ureide analysis.
E: 200 μL of the reaction solution was put into a well of microplate.
F: Example of amide analysis in a microplate.

***Field and planting conditions.***

The experiment was carried out in an upland field located in Nagaoka (Nagakura field) in the Niigata Agricultural Research Institute, Japan, which had been converted from a drained paddy field in the previous year. The soil was a Fine-textured Gray Lowland soil (gray type). A basal application of N (ammonium sulfate, 16 kg N $ha^{-1}$), P (superphosphate, 60 kg $P_2O_5$ $ha^{-1}$), K (potassium chloride, 80 kg $K_2O$ $ha^{-1}$) and Ca (calcium hydroxide, 1,000 kg $Ca(OH)_2$ $ha^{-1}$) fertilizers was carried out in the plow layer at a depth of approximately 0-10 cm in all experimental plots. The seeds were sown using a single-stem training, and the seeding rate was 8.9 plants $m^{-2}$ (row space 75 cm x planting distance 15 cm), which is the conventional planting density in Niigata Prefecture. Seeds of soybean (cv. Enrei) were used for the experiment. Before planting, the seeds were dipped for approximately 15 min in a diluted culture of *Bradyrhizobium japonicum* (strain USDA 110) (1 $x10^9$ cells $mL^{-1}$).

***Experiment 1: Effect of excluding N, P, K, and Ca nutrients from fertilizer***

Five treatments were conducted with three replications; 1) control: treated with all nutrients, 2) -N: treated without ammonium sulfate, 3) -P: treated without superphosphate, 4) -K: treated without potassium chloride, 5) -Ca: treated without calcium hydroxide. Seeds were planted on 4$^{th}$ June, 2007. Root bleeding xylem sap and an apoplast fluid were collected on 24$^{th}$ July (R1 stage), on 27$^{th}$ August (R5 stage) and on 25$^{th}$ September (R7 stage). The volume of each solution was determined by weighing the Eppendorf tube, and the

concentrations of ureide-N, amide-N, and nitrate-N were measured using 2.5 μL of solution by micro-analysis as described above.

***Experiment 2: Effect of excluding N, P, and K nutrients from fertilizer***

Five treatments were conducted with three replications; 1) control: treated with all nutrients, 2) -N: treated without ammonium sulfate, 3) -P: treated without superphosphate, 4) -K: treated without potassium chloride. In 2008 Ca was not applied in all plots. Seeds were planted on 27th May, 2008. Root bleeding xylem sap and apoplast fluid were collected on 22th July at R1 stage, on 25th August at R5 stage, and on 25th September at R7 stage. The volume of each solution was determined, and the concentrations of amide-N, nitrate-N and ureide-N were measured using 2.5 μL of solution by micro-analysis as described above.

***Experiment 3: Effect of top dressing of N fertilizer at initial flowering stage.***

Four treatments were conducted; 1) control: treated without top-dressing of N fertilizer, 2) 2N: treated with top dressing of urea (2 kg N $10a^{-1}$) at initial flowering stage (R1), 3) 5N: treated with top dressing of urea (5 kg N $10a^{-1}$), 4) 10N: treated with top dressing of urea (10 kg N $10a^{-1}$). In 2009 Ca was not applied in all plots. Seeds were planted on 28th May, 2009. Root bleeding xylem sap and apoplast fluid were collected on 21th July at R1 stage, on 24th August at R5 stage, and on 24th September at R7 stage. The volume of each solution was determined, and the concentrations of ureide-N, amide-N, and nitrate-N were measured using 2.5 μL of solution by the micro-analysis as described above.

***Experiment 4: Effect of sampling time on the concentration of ureide-N, amide-N, and nitrate-N at flowering stage.***

Seeds were planted on 27th May, 2008 as same as Experiment 2. Root bleeding xylem sap and apoplast fluid were collected at 10, 12, 14 and 16 AM on 22th July (R1 stage). The volume of each solution was determined, and the concentrations of ureide-N, amino-N, and nitrate-N were measured using 2.5 μL of solution by the micro-analysis.

***Experiment 5: Effect of sampling gravity on the concentration of amide-N, nitrate-N and ureide-N in stem apoplastic fluid at flowering stage.***

Seeds were planted on 27th May, 2008 as same as Experiment 2. Stem apoplast fluid was collected at 500, 1000, 2200, and 5000 x G on 22th July (R1 stage). The volume of each solution was determined, and the concentrations of ureide-N, amide-N, and nitrate-N were measured using 2.5 μL of solution by micro-analysis.

**Results and discussion**

***Micro-scale analysis of ureide-N, amide-N, and nitrate-N***

Figure 6 shows standard calibrations for the microscale analysis of the concentration of ureides, amides, and nitrate. The absorbance of blank was subtracted from the sample absorbance. Duplicated analysis was carried out for each concentration, and the average values are shown. The coefficients of determination for the microanalysis of ureides, amides, and nitrate were 0.994, 0.999, and 0.999. This indicates the accuracy of the microanalysis is as high as conventional method using 50 μL of sample solution for each analysis.

***Amount of root bleeding xylem sap and stem fluid collected by centrifuge.***

Figures 7, 8, 9 show the weight of xylem sap (left) and stem fluid collected by centrifuge in experiment 1, 2, and 3, respectively. In Exp.1, the weight of xylem sap were not significantly different among fertilizer treatments. The average weights of xylem sap were 1633 mg, 1590 mg and 127 mg for R1, R5 and R7 stages. The volume of sap collected at R7 was very low. On the other hand, the weight of stem fluid can be collected any stages and any treatment, although the average weights are 22.5 mg, 38.2 mg and 69.0 mg at R1, R5 and R7 stages. These trends are similar in experiment 2 in 2008. We could not collect any xylem sap at R5 stage in experiment 3 (Figure 7). Although the weight of stem fluid was much lower than xylem sap, but it is always obtained at

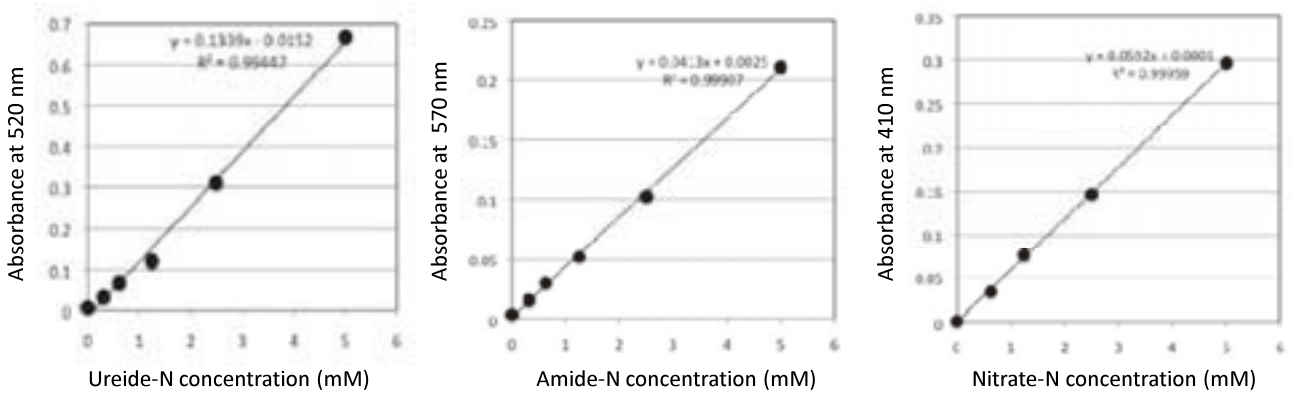


**Fig 6.** Calibration of amide-N, nitrate-N and ureide-N analysis.

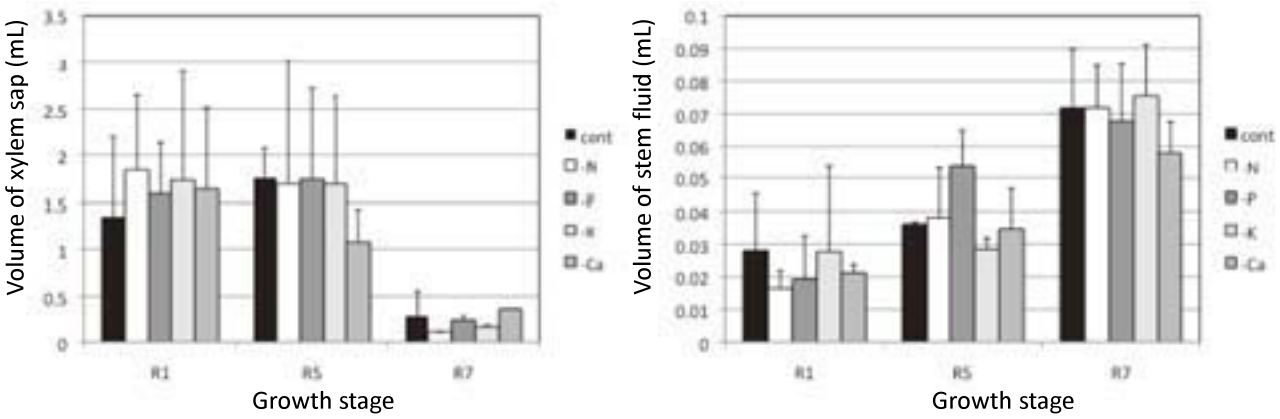


**Fig 7.** Volume of xylem sap (left) and stem fluid (right) collected at R1, R5 and R7 stages in Experiment 1.

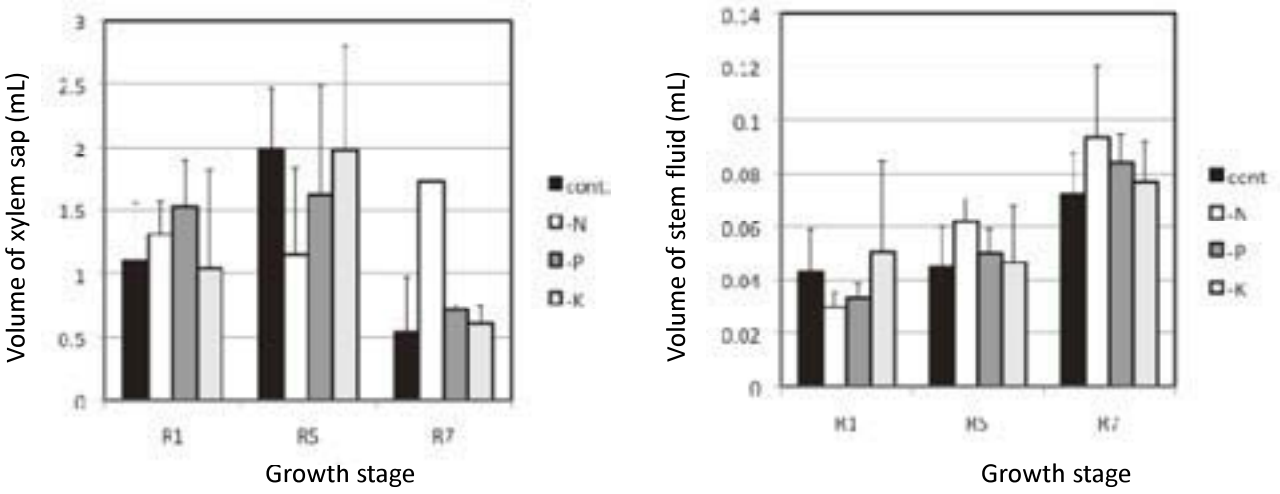


**Fig 8.** Volume of xylem sap (left) and stem fluid (right) collected at R1, R5 and R7 stages in Experiment 2.

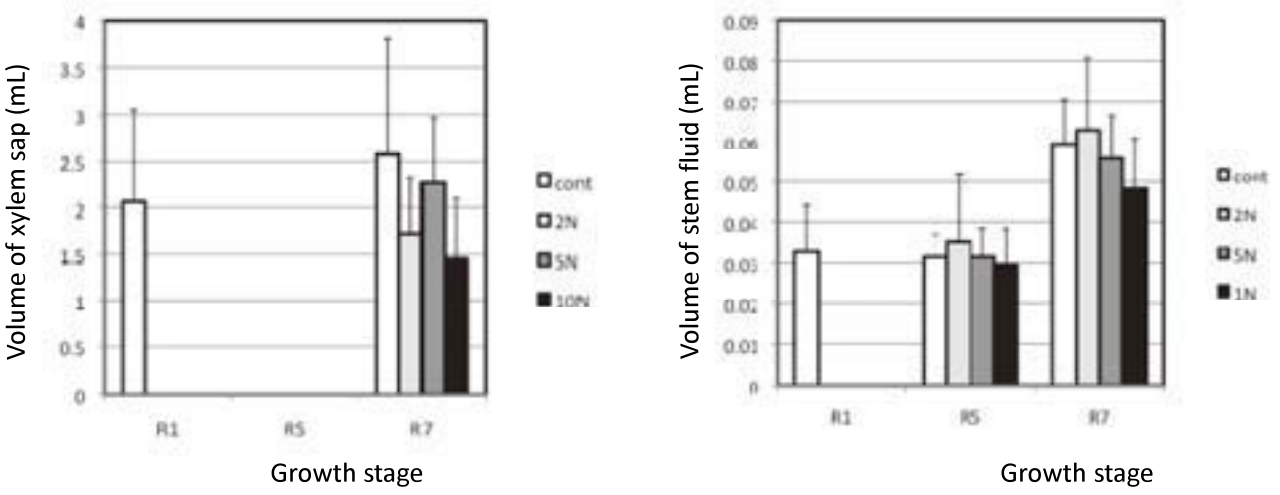


**Fig 9.** Volume of xylem sap (left) and stem fluid (right) collected at R1, R5 and R7 stages in Experiment 3.

any stage and tended to increase from R1 to R7.

***Comparison of the relative ureide-N percent between xylem sap and stem fluid.***

Figure 10, 11, and 12 show the comparison between relative ureide-N percent between xylem sap (left) and stem fluid (right) in Exp. 1, 2, and 3 respectively. The relative ureide-N percent analyzed by xylem sap was high at R1 and R5, about 80-90% but became low about 60% at R7. On the other hand, the relative ureide-N percent was lower about 50-60% at R1 stage, and it was constant until R7 stage. From these data, it was concluded that stem fluid are not used for relative ureide-N analysis at R1 and R5 stages.

Table 1, 2, and 3 shows the concentrations of amide, nitrate and ureides in xylem sap (left) and stem fluid (right) obtained by Exp. 1, 2, and 3 respectively. The concentrations

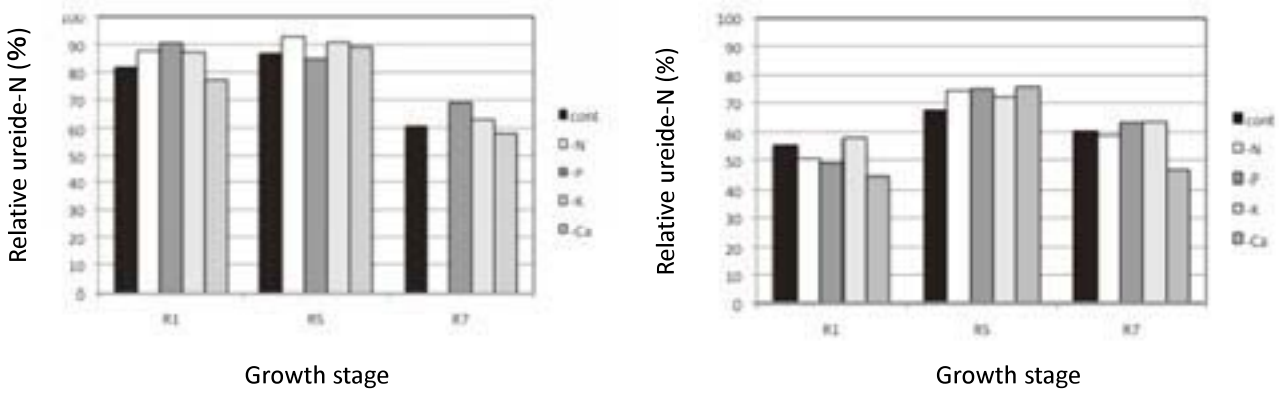


**Fig 10.** Relative ureide-N % calculated from xylem sap (left) and stem fluid (right) collected at R1, R5 and R7 stages in Experiment 1.

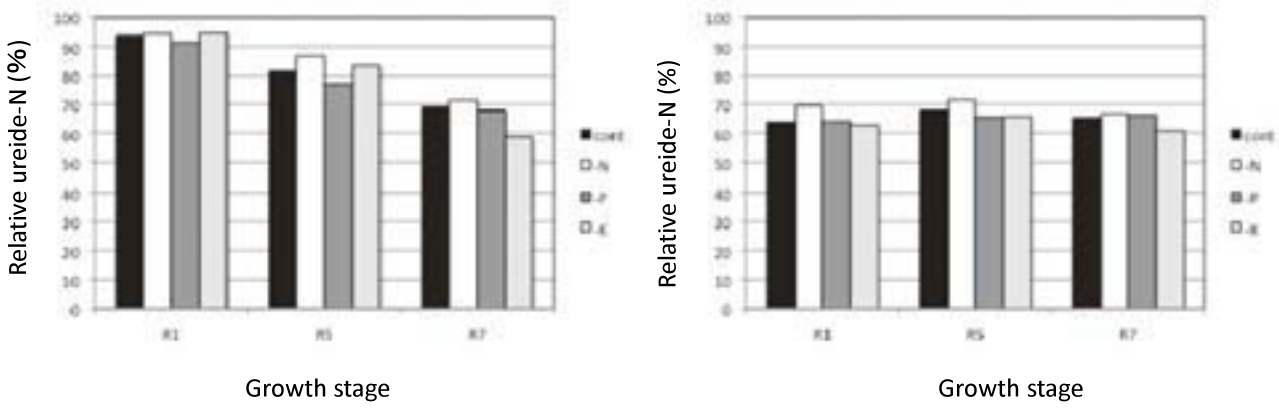


**Fig 11.** Relative ureide-N % calculated from xylem sap (left) and stem fluid (right) collected at R1, R5 and R7 stages in Experiment 2.

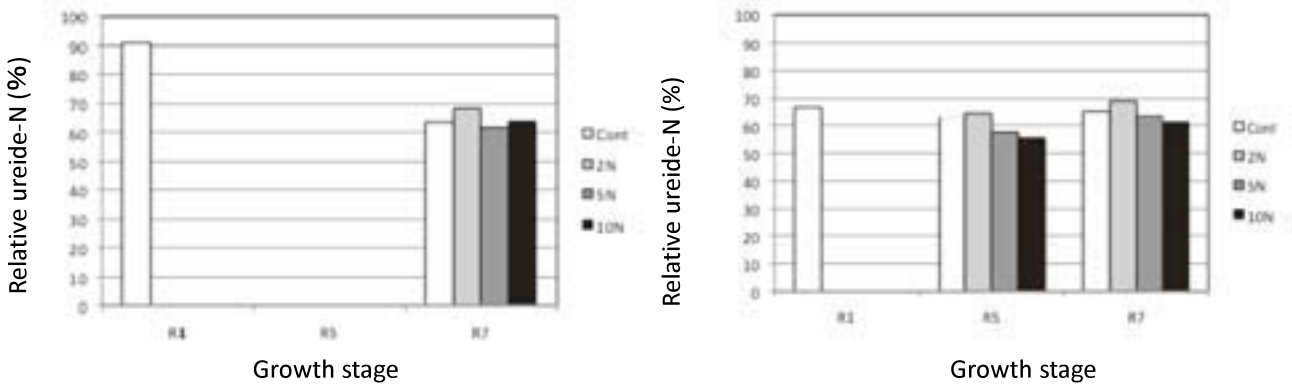


**Fig 12.** Relative ureide-N % calculated from xylem sap (left) and stem fluid (right) collected at R1, R5 and R7 stages in Experiment 3.

**Table 1.** Concentration of amide, nitrate and ureide in xylem sap (left) and in stem fluid (right) and the relative ureide percent cultivated with different fertilization treatments in 2007.

| Xylem Sap | | | | | | Stem Fluid | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Amide Concentration (mM) | | | | | | Amide Concentration (mM) | | | | | |
| Stage | Cont | -N | -P | -K | -Ca | Stage | Cont | -N | -P | -K | -Ca |
| R1 | 0.82 (0.55) | 0.45 (0.20) | 0.63 (0.12) | 0.49 (0.11) | 1.05 (0.46) | R1 | 2.15 (0.86) | 3.88 (1.60) | 4.58 (0.92) | 1.96 (0.18) | 3.15 (1.21) |
| R5 | 3.27 (2.21) | 1.78 (0.71) | 3.26 (1.27) | 2.66 (1.87) | 3.46 (2.23) | R5 | 15.70 (3.19) | 12.98 (6.59) | 6.97 (1.73) | 17.15 (3.23) | 8.99 (2.63) |
| R7 | 5.8* | ND** | 4.87 (0.01) | 7.59 (0.76) | 5.48* | R7 | 3.65 (1.52) | 5.10 (2.86) | 5.46 (0.73) | 5.01 (0.72) | 6.71 (0.81) |
| Nitrate Concentration (mM) | | | | | | Nitrate Concentration (mM) | | | | | |
| Stage | Cont | -N | -P | -K | -Ca | Stage | Cont | -N | -P | -K | -Ca |
| R1 | 0.97 (0.26) | 1.66 (1.36) | 0.82 (0.40) | 1.79 (0.64) | 1.25 (0.76) | R1 | 0.93 (0.76) | 1.50 (0.40) | 1.97 (0.74) | 1.58 1.65) | 1.91 (1.23) |
| R5 | 0.15 (0.04) | 0.11 (0.17) | 0.02 (0.02) | 0.03 (0.03) | 0.11 (0.08) | R5 | 1.11 (0.42) | 1.16 (0.49) | 0.61 (0.13) | 1.22 (0.13) | 1.05 (0.53) |
| R7 | 0.93* | ND** | 0.72 (0.10) | 1.08 (0.09) | 2.04* | R7 | 0.93 (0.33) | 0.99 (0.49) | 0.66 (0.27) | 0.63 (0.23) | 0.93 (0.40) |
| Ureide Concentration (mM) | | | | | | Ureide Concentration (mM) | | | | | |
| Stage | Cont | -N | -P | -K | -Ca | Stage | Cont | -N | -P | -K | -Ca |
| R1 | 2.70 (1.28) | 4.73 (2.53) | 5.34 (1.50) | 4.77 (0.37) | 2.83 (0.72) | R1 | 1.89 (1.56) | 2.48 (1.39) | 2.69 (0.90) | 1.84 (0.05) | 1.63 (0.99) |
| R5 | 9.90 (3.94) | 11.45 (2.45) | 9.78 (5.53) | 12.08 (3.12) | 13.64 (9.14) | R5 | 16.95 (4.09) | 19.47 (8.26) | 10.90 (1.70) | 22.81 (2.00) | 14.60 (1.38) |
| R7 | 4.83* | ND** | 6.04 (1.84) | 6.92 (0,.28) | 4.49* | R7 | 3.00 (0.43) | 3.80 (1.42) | 5.16 (1.31) | 4.68 (0.54) | 3.40 (1.76) |
| Relative ureide-N percent (%) | | | | | | Relative Ureide-N percent (%) | | | | | |
| Stage | Cont | -N | -P | -K | -Ca | Stage | Cont | -N | -P | -K | -Ca |
| R1 | 81.7 (3.4) | 87.7 (7.8) | 90.6 (3.7) | 87.2 (3.7) | 77.2 (0.5) | R1 | 55.3 (11.2) | 50.3 (8.1) | 48.8 (2.7) | 57.9 (6.0) | 44.1 (15.2) |
| R5 | 86.7 (4.1) | 92.7 (1.0) | 84.9 (3.4) | 90.8 (5.9) | 89.1 (3.5) | R5 | 67.3 (2.4) | 74.4 (7.1) | 75.1 (4.0) | 72.1 (2.3) | 75.8 (4.0) |
| R7 | 60.6* | ND** | 69.1 (1.1) | 63.0 (1.1) | 58* | R7 | 60.3 (5.7) | 58.8 (4.8) | 63.5 (5.2) | 63.7 (6.2) | 46.3 (16.5) |

Values are expressed as "Average (Standard deviation)", * One datum, ** No data

**Table 2.** Concentration of amide, nitrate and ureide in xylem sap (left) and in stem fluid (right) and the relative ureide percent cultivated with different fertilization treatments in 2008.

| Xylem Sap | | | | | Stem Fluid | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Amide Concentration (mM) | | | | | Amide Concentration (mM) | | | | |
| Stage | Cont | -N | -P | -K | Stage | Cont | -N | -P | -K |
| R1 | 1.07 (0.24) | 0.71 (0.23) | 0.82 (0.25) | 0.78 (0.22) | R1 | 7.34 (0.96) | 6.91 (1.46) | 6.95 (1.39) | 7.61 (1.58) |
| R5 | 4.85 (1.48) | 4.31 (1.15) | 5.70 (2.15) | 3.46 (0.91) | R5 | 11.40 (0.98) | 11.68 (1.47) | 12.06 (2.48) | 12.23 (1.72) |
| R7 | 4.10 (1.22) | 4.56* | 3.03 (1.08) | 3.79 (2.70) | R7 | 4.29 (1.02) | 4.40 (1.83) | 4.09 (1.09) | 4.83 (2.00) |
| Nitrate Concentration (mM) | | | | | Nitrate Concentration (mM) | | | | |
| Stage | Cont | -N | -P | -K | Stage | Cont | -N | -P | -K |
| R1 | 1.54 (0.45) | 1.82 (0.52) | 2.10 (0.35) | 1.57 (0.98) | R1 | 8.86 (1.23) | 5.21 (0.53) | 7.83 (2.32) | 8.90 (0.94) |
| R5 | 3.77 (0.58) | 2.84 (0.43) | 3.74 (0.87) | 3.78 (1.39) | R5 | 4.51 (0.92) | 3.77 (1.77) | 3.94 (1.15) | 4.37 (1.32) |
| R7 | 1.77 (0.71) | 1.52* | 2.50 (0.05) | 1.49 (0.52) | R7 | 1.21 (0.20) | 1.29 (0.57) | 1.88 (0.28) | 1.47 (0.76) |
| Ureide Concentration (mM) | | | | | Ureide Concentration (mM) | | | | |
| Stage | Cont | -N | -P | -K | Stage | Cont | -N | -P | -K |
| R1 | 13.76 (0.90) | 13.82 (2.09) | 9.88 (0.27) | 13.85 (3.26) | R1 | 10.35 (0.86) | 10.88 (1.23) | 9.77 (1.79) | 10.17 (1.08) |
| R5 | 14.87 (2.65) | 19.19 (4.50) | 12.63 (3.10) | 13.55 (3.96) | R5 | 14.59 (2.45) | 17.16 (2.74) | 13.31 (1.68) | 13.94 (2.07) |
| R7 | 5.60 (1.03) | 6.6* | 4.66 (1.37) | 3.16 (0.71) | R7 | 4.68 (1.24) | 4.98 (0.50) | 5.00 (1.31) | 4.27 (1.29) |
| Relative ureide-N percent (%) | | | | | Relative ureide-N percent (%) | | | | |
| Stage | Cont | -N | -P | -K | Stage | Cont | -N | -P | -K |
| R1 | 93.7 (0.5) | 94.4 (1.2) | 91.4 (1.6) | 94.6 (0.46) | R1 | 63.7 (3.7) | 69.4 (5.1) | 64.1 (2.7) | 62.8 (2.0) |
| R5 | 81.3 (3.9) | 86.7 (3.6) | 77.0 (4.2) | 83.3 (3.3) | R5 | 67.8 (4.1) | 71.5 (4.7) | 65.5 (6.1) | 65.7 (4.0) |
| R7 | 68.9 (7.68) | 71.2* | 67.8 (12.0) | 58.5 (15.7) | R7 | 65.3 (5.1) | 66.8 (7.3) | 66.4 (2.6) | 60.7 (0.7) |

Values are expressed as "Average (Standard deviation)", * One datum

**Table 3.** Concentration of amide, nitrate and ureide in xylem sap (left) and in stem fluid (right) and the relative ureide percent cultivated with different fertilization treatments in 2009.

| Xylem Sap | | | | | Stem Fluid | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Amide Concentration (mM) | | | | | Amide Concentration (mM) | | | | |
| Stage | Cont | 2N | 5N | 10N | Stage | Cont | 2N | 5N | 10N |
| R1 | 1.27 (0.26) | | | | R1 | 6.31 (1.41) | | | |
| R5 | ND** | ND | ND | ND | R5 | 11.60 (0.79) | 12.01 (1.47) | 11.93 (1.62) | 11.92 (1.06) |
| R7 | 4.56 (0.99) | 3.88 (1.22) | 4.15 (1.08) | 4.67 (1.30) | R7 | 4.64 (1.16) | 3.85 (1.21) | 4.90 (0.84) | 5.37 (1.53) |
| Nitrate Concentration (mM) | | | | | Nitrate Concentration (mM) | | | | |
| Stage | Cont | 2N | 5N | 10N | Stage | Cont | 2N | 5N | 10N |
| R1 | 1.93 (0.96) | | | | R1 | 5.98 (1.00) | | | |
| R5 | ND | ND | ND | ND | R5 | 5.45 (1.75) | 4.73 (1.79) | 6.26 (1.21) | 6.58 (1.67) |
| R7 | 1.82 (0.44) | 1.93 (0.36) | 1.98 (0.51) | 2.29 (0.43) | R7 | 1.12 (0.23) | 1.26 (0.19) | 1.24 (0.25) | 1.19 (0.28) |
| Ureide Concentration (mM) | | | | | Ureide Concentration (mM) | | | | |
| Stage | Cont | 2N | 5N | 10N | Stage | Cont | 2N | 5N | 10N |
| R1 | 10.99 (2.52) | | | | R1 | 9.49 (1.73) | | | |
| R5 | ND | ND | ND | ND | R5 | 12.43 (2.06) | 13.44 (2.46) | 10.38 (2.24) | 9.71 (2.41) |
| R7 | 4.90 (1.20) | 5.23 (0.70) | 4.18 (0.93) | 5.13 (0.80) | R7 | 5.09 (1.37) | 5.15 (1.43) | 4.98 (1.37) | 4.83 (1.41) |
| Relative ureide-N percent (%) | | | | | Relative Ureide-N percent (%) | | | | |
| Stage | Cont | 2N | 5N | 10N | Stage | Cont | 2N | 5N | 10N |
| R1 | 90.7 (2.1) | | | | R1 | 67.0 (4.8) | | | |
| R5 | ND | ND | ND | ND | R5 | 63.2 (5.0) | 64.8 (4.4) | 57.5 (6.9) | 55.4 (5.5) |
| R7 | 63.5 (7.7) | 68.5 (5.7) | 61.6 (9.8) | 63.7 (8.5) | R7 | 65.4 (9.5) | 69.2 (7.6) | 63.7 (6.1) | 61.1 (10.4) |

Values are expressed as "Average (Standard deviation)",　** No data

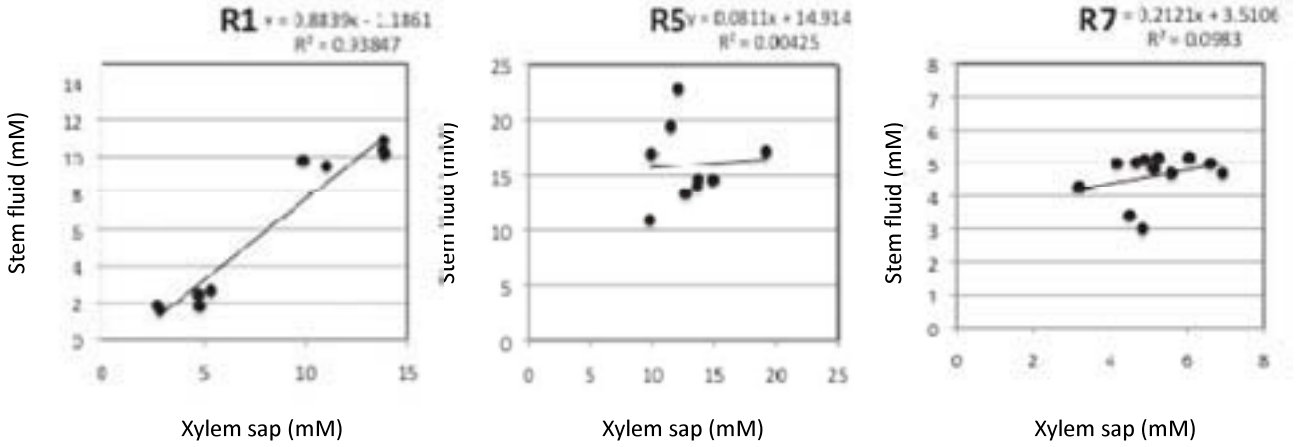


**Fig 13.** Correlation of ureide-N concentration between xylem sap and stem fluid collected at R1, R5 and R7 stages in Experiment 1, 2 and 3.

of amide-N in stem fluid were much higher than those in xylem sap at R1 and R5 stages, although those are comparable at R7. In addition, the concentration of nitrate-N at R5 in Exp. 1 and at R1 in Exp. 2 were higher in the stem fluid compared with xylem sap. The level of ureide-N in xylem sap and stem fluid are comparable at all the stages and treatments. Therefore, the lower relative ureide-N percentage in stem fluid at R1 and R5 is due to the higher concentration of amide-N and nitrate-N (Figure 13, 14, 15). At R7 stage the concentrations of amide-N, nitrate-N and ureide-N were similar between root bleeding xylem sap and stem fluid, therefore, the relative ureide-N percent was comparable at R7 stage (Figure 14).

***Effect of time of day for collecting xylem sap and stem fluid.***

Table 4 shows the effect of time of day for collecting xylem sap (left) and stem fluid (right). The weight of xylem sap was the highest (1040 mg) at 10 AM and gradually decreased to 276 mg. The concentration of the nitrate amide and ureide also tended to decrease from 10 AM to 16 AM. However, the relative ureide-N percent was constant at 88-89% irrespective of time. This data shows that we can correct xylem sap at any time for estimating the relative ureide percent from morning to afternoon. The weight of stem fluid did not decrease from 10 AM to 16 AM, but the concentrations of nitrate, amides and ureides tended to

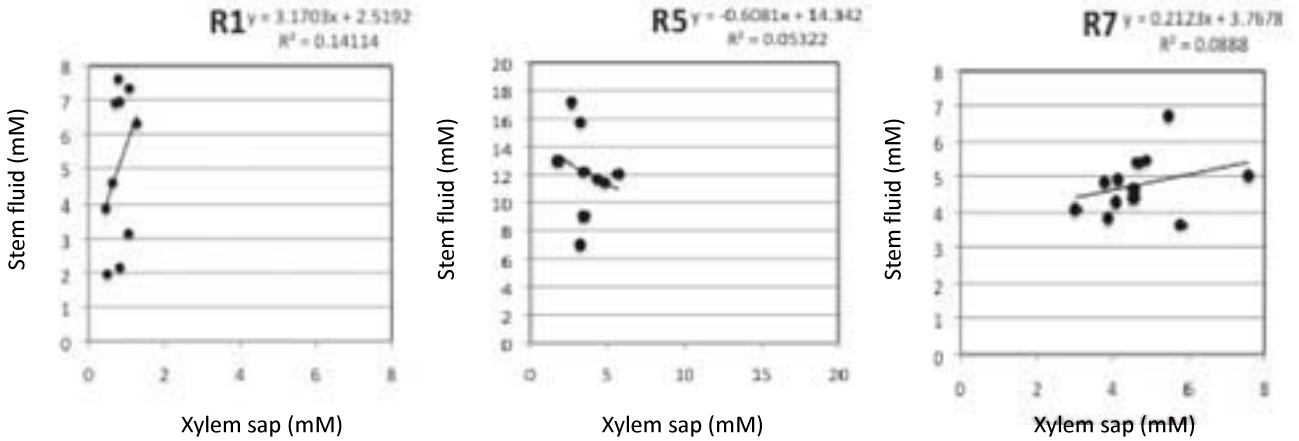


**Fig 14.** Correlation of amide-N concentration between xylem sap and stem fluid collected at R1, R5 and R7 stages in Experiment 1, 2 and 3.

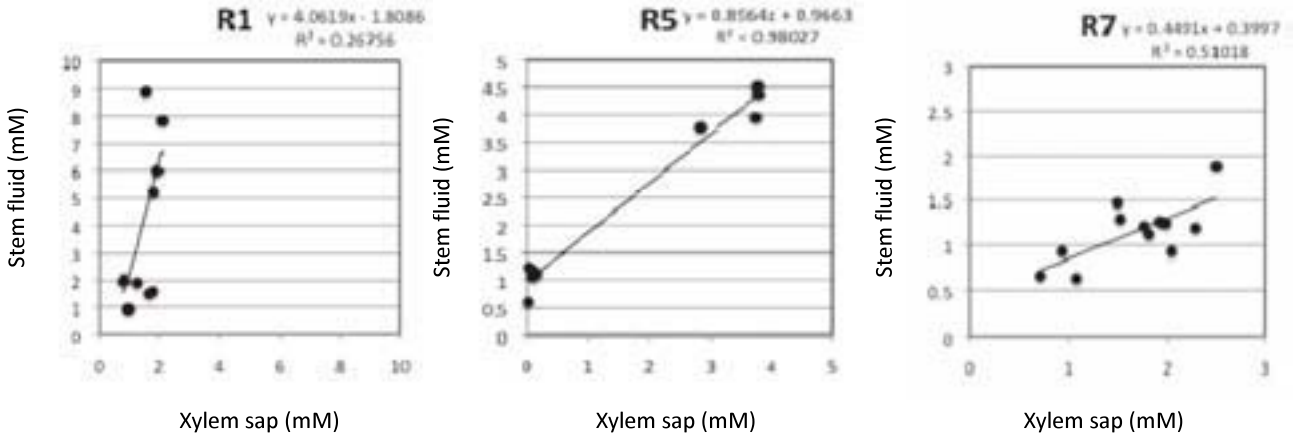


**Fig 15.** Correlation of nitrate-N concentration between xylem sap and stem fluid collected at R1, R5 and R7 stages in Experiment 1, 2 and 3.

**Table 4.** Concentration of amide, nitrate and ureide in xylem sap (left) and in stem fluid (right) and the relative ureide percent collected with different time of the day in 2009.

| Xylem Sap | | | | | Stem Fluid | | | |
|---|---|---|---|---|---|---|---|---|
| Time of the day (AM) | 10 | 12 | 14 | 16 | 10 | 12 | 14 | 16 |
| liquid(mg) | 1040 (541) | 780 (380) | 556 (65) | 279 (171) | 39.1 (16.9) | 21.2 (2.8) | 35.4 (6.1) | 49.7 (6.0) |
| Nitrate concentration(mM) | 0.98 (0.44) | 0.87 (0.36) | 1.18 (1.07) | 0.60 (0.20) | 6.33 (1.79) | 9.65 (1.41) | 3.21 (0.15) | 3.50 (0.72) |
| Amide concentration (mM) | 2.45 (0.30) | 2.16 (0.52) | 1.81(0.35) | 1.28 (0.24) | 14.67 (4.27) | 13.46 (3.96) | 7.91 (1.87) | 9.44 (2.25) |
| Ureide concentration (mM) | 11.00 (1.60) | 9.86 (2.15) | 9.26 (2.60) | 7.06 (1.80) | 7.65 (2.16) | 7.82 (0.78) | 3.31 (0.69) | 4.02 (0.89) |
| Relaive Ureide-N (%) | 88.1 (1.7) | 88.1 (3.2) | 88.5 (2.0) | 89.4 (3.8) | 46.1 (8.0) | 46.3 (5.2) | 41.0 (2.5) | 42.0 (6.5) |

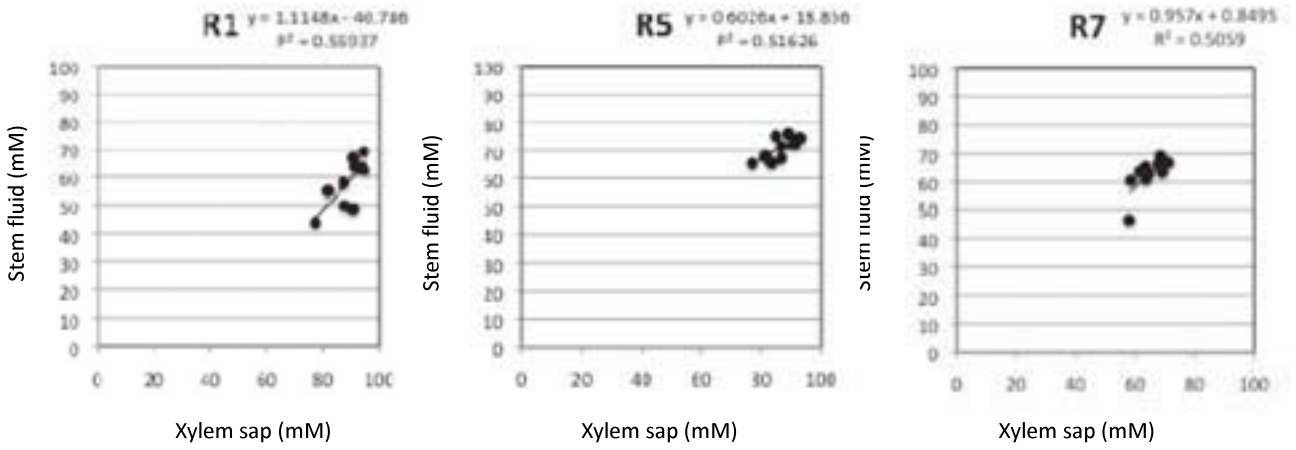


**Fig 16.** Correlation of relative ureide-N % between xylem sap and stem fluid collected at R1, R5 and R7 stages in Experiment 1, 2 and 3.

**Table 5.** The effect of gravity for collecting stem fluid on volume, and concentrations of nitrate, amide and ureide, and relative ureide percent.

| | 500 xG | 1000 xG | 2200 xG | 5000 xG |
|---|---|---|---|---|
| Volume (mg) | 30.6 (4.6) | 34.9 (11.8) | 36.4 (10.0) | 50.3 (12.4) |
| Nitrate (mM) | 3.78 (0.44) | 2.97 (0.28) | 4.08 (0.34) | 5.91 (1.26) |
| Amide (mM) | 8.48 (2.10) | 9.25 (2.16) | 9.38 (0.44) | 12.76 (2.92) |
| Ueide (mM) | 4.38 (0.53) | 5.14 (1.19) | 4.22 (0.49) | 9.82 (1.78) |
| RU (%) | 46.1 (4.7) | 48.8 (5.4) | 42.4 (2.9) | 55.4 (3.4) |

Average (standard deviation)

decrease. The relative ureide-N percent was relatively constant from 41-46%.

***Effect of gravity for collecting stem fluid on weight and nitrate, amides, and ureides concentrations.***

Table 5 shows the effect of gravity for collecting stem fluid on weight and concentrations of nitrate, amides and ureides in the fluid. The fluid weights were similar about 30-36 mg among 500, 1000 and 2200 x G, but it was higher about 50 mg at 5000 x G. In addition, the concentration of nitrate, amide and ureide tended to be higher at 5000 x G compared with lower gravities. We collected stem fluid at 2200 x G, and this is similar to the lower gravity as 500 and 1000 x G. However, when the stem fluid was collected at 5000 x G, the weight and concentrations of nitrate, amide, and ureides increased, suggesting that cells possibly vacuole may be destroyed and released the fluid in the vacuole was also collected at this gravity.

## Discussion

***Advantage of microanalysis of relative ureide assay***

The advantage of micro-analysis of amide-N, ureide-N, and nitrate-N for relative ureide assay is as follows.

Researchers can save the time for each analysis because we can use a lot of tubes for reaction at once, and colorimetric analysis is rapid by using micro-plate. Using a conventional method, about 20-50 samples can be analyzed one day, but we can analyze about 200 samples per day using this micro-analysis. When root bleeding xylem sap is transparent and colorless, the analysis for the blank is not necessary. The accuracy may depend on the pipetting, and duplicated analysis for standard solution and samples may be better for accurate measurement.

Due to the use of the small amount of reagent 1/20 compared with a conventional method, we can save the cost for purchasing the reagents, and disposal of the reagents. Sato et al. (1998) reported that allantion, allantoic acid, aspartagine and nitrate can be analyzed by capillary electrophoresis. This method can be applied for small amount of xylem sap, but it needs longer time than the micro-analysis when sample number is large.

***The use of stem fluid collected by centrifuge.***

We could succeed to collect stem fluid by centrifuge at any stage from R1 to R7. In addition, the volumes of the stem fluid were higher in late stage than early stage, when the bleeding of xylem sap from root is difficult to obtain. However, the stem fluid collected at R1 and R5 contained a higher percentage of amide-N, and consequently exhibited a

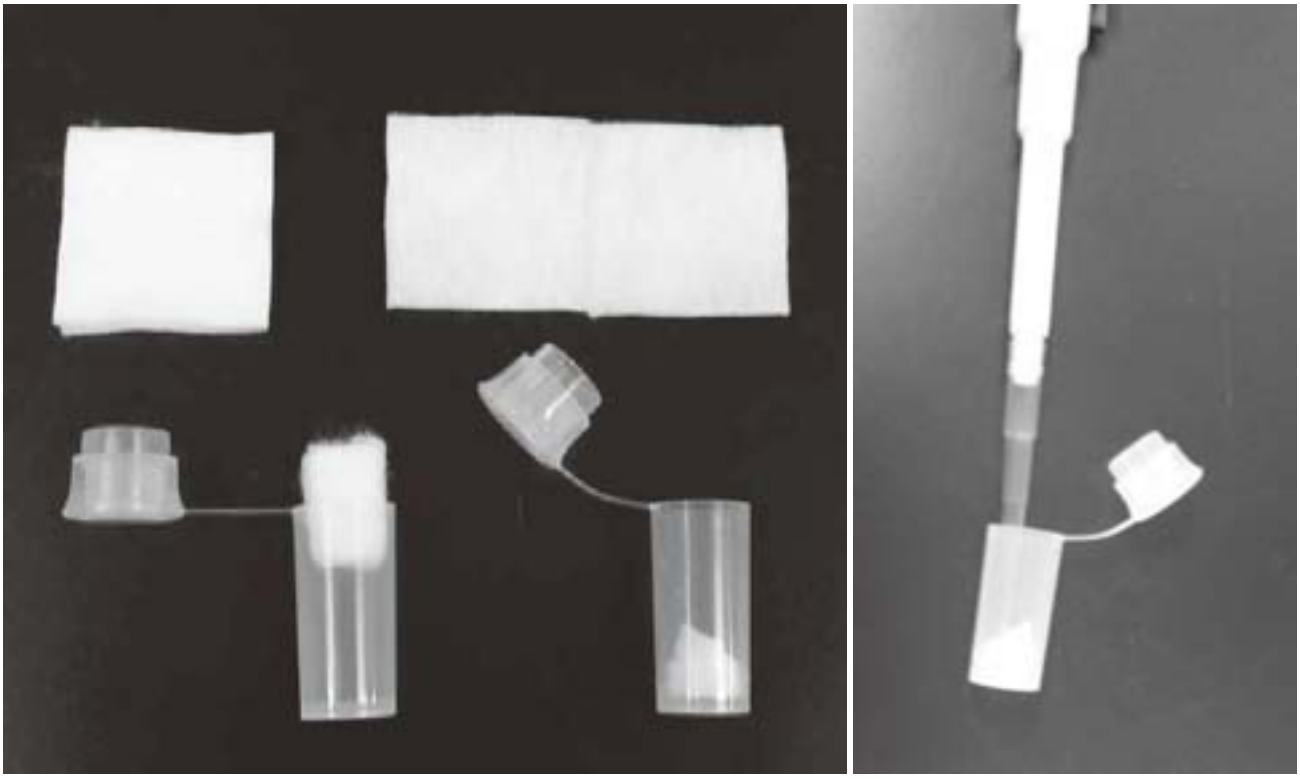

**Fig 17.** Sampling tube with cotton on the cut stem.

lower %Ndfa. Therefore, we cannot use the stem fluid instead of root bleeding xylem sap at these stages. However, at R7 stage, the concentrations of ureide-N, amide-N and nitrate-N were comparable between root bleeding xylem sap and stem fluid. Therefore, it is possible to use this method at R7 stage.

By collection of stem fluid, both the apoplast solution, which exist in the apoplast of stem outside of xylem vessels and xylem solution might be collected by centrifugation (Figure 3). In early stages such as R1 and R5 the apoplast solution in stem contain a higher concentration of amides than xylem sap. On the other hand at R7, the apoplast solution in the stem contain the same concentration of each components in xylem sap.

A tube with cotton fiber have been used by the alternative way to collect xylem sap (Nohara et al. 2005, Nagumo et al. 2010). Figure 17 shows an example of the tube with cotton. At later stages such as R7 the surface of the stem tend to be rough and xylem sap leaks after it bleeds. Using the tube with cotton, the bleeding xylem sap can be hold in a cotton by capillary pressure. The xylem sap can be recovered by pipetting the sap from cotton.

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# 導管液と茎アポプラスト液の微量相対ウレイド分析によるダイズの窒素固定依存率の推定

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**要　約**

相対ウレイド法は、圃場栽培ダイズの窒素固定依存率を推定するための簡便で信頼のできる方法である。一般に主茎株切断面から溢泌する導管液中の硝酸、アミド、ウレイド濃度を分光光度計を用いて比色定量する。我々は、通常の分析で用いる導管液量50 μL の代わりに、その1/20の2.5 μL を用いる微量分析法を開発した。呈色反応は、1.5 mL エッペンドルフチューブ内で行ない、呈色液200 μL を96穴マイクロプレートのウェルに入れ、マイクロプレートリーダーで吸光度を測定した。標準液を２連で測定することにより、精度の高い検量線の作成が可能であった。本分析法を用いることにより、1日に200試料を測定できた。また、生育後期（黄葉期 R7）や、土壌が乾燥すると、導管液を溢泌しないことがあるため、確実に試料を採取する方法として、携帯型遠心器（2,200 x G, 10秒）を用いて茎のアポプラスト液を採取した。茎は、主茎下部から2, 3cm の長さで切り出し、1.5 mL のエッペンドルフチューブに入れて回収した。茎のアポプラスト液は、時期や気象条件によらず株あたり30-50 mg程度回収できた。各窒素成分濃度および相対ウレイドパーセントを比較したところ、R7では、導管液とほぼ同様の値が得られ、導管液の代わりに利用できる可能性が示された。しかしながら、R1, R5では、アポプラスト液のアミド，硝酸濃度が導管液よりも高く、相対ウレイドパーセントは、50%程度と導管液80-90%と異なる値となった。

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