2. Conventional Methods

2.1. N-fixing activity

2.1.1. Acetylene reduction assay

Nitrogenase reduces acetylene (C_2H_2) to ethylene (C_2H_4), and the ethylene formed can be very sensitively detected by gas chromatography. The acetylene reduction assay (ARA) is the most sensitive method to elucidate biological N_2 fixation activity. It could be used to compare nitrogenase activities among treatments in laboratory experiments as well as in field experiments. However, the ARA cannot be quantitatively converted to the amount of N_2 fixed. It should be noted that the ARA does not relate directly to N_2 fixation, but instead measures electron flux through nitrogenase. Total electron allocation for both N_2 fixation and H_2 evolution will be measured by ARA. In addition, ARA is difficult to be applied in field experiment mainly due to difficulty of full recovery of nodulated root system.

The standard ARA method involves enclosed detached nodules or nodulated root systems in air tight containers and exposing them to an atmosphere containing C_2H_2 (Peoples and Herridge 1990). However, detached nodules are ready to desiccate and decrease N_2 fixation activity.

For hydroponically cultivated legume plants, the ARA can be measured in intact plants in a sealed pot (Fig. 1A). About 10 % of the air volume inside is replaced by pure acetylene. After incubation in a short period for 15 min or 30 min, 0.5 mL of the gas inside is sampled using a hypodermic syringe, and the gas is analyzed by GC (gas chromatography) equipped with FID detector. The column is Porapack N (GL Sciences). About 99.5 % standard ethylene was diluted to 1000 times and 0.5mL was injected.

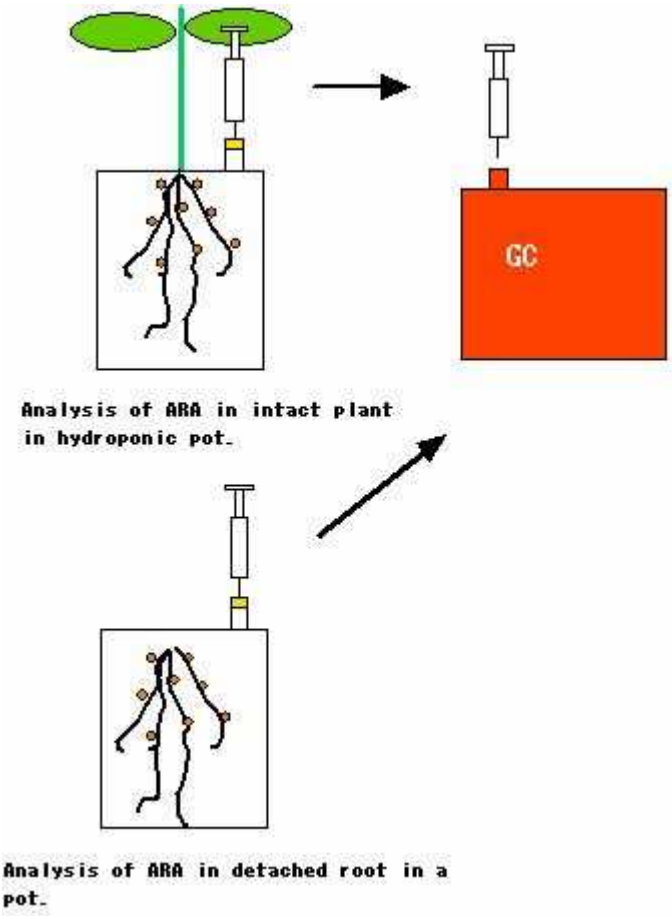


Fig.1A: ARA measurement of nodulated legumes in intact plants and detached roots

Detached nodulated roots are most frequently used for ARA analysis (Fig.1B). Detached root system is put in a glass jar (700 mL) and 10 % of the gas inside is replaced by acetylene. After 20 min incubation at 25℃, 0.5 mL of gas inside the jar was taken and concentration of ethylene is analyzed. After ARA

measurement, roots are dried and nodule DW is measured. ARA is expressed as μmole ethylene formed per hr per plant or μmole ethylene formed per hr per g DW of nodules (specific ARA).

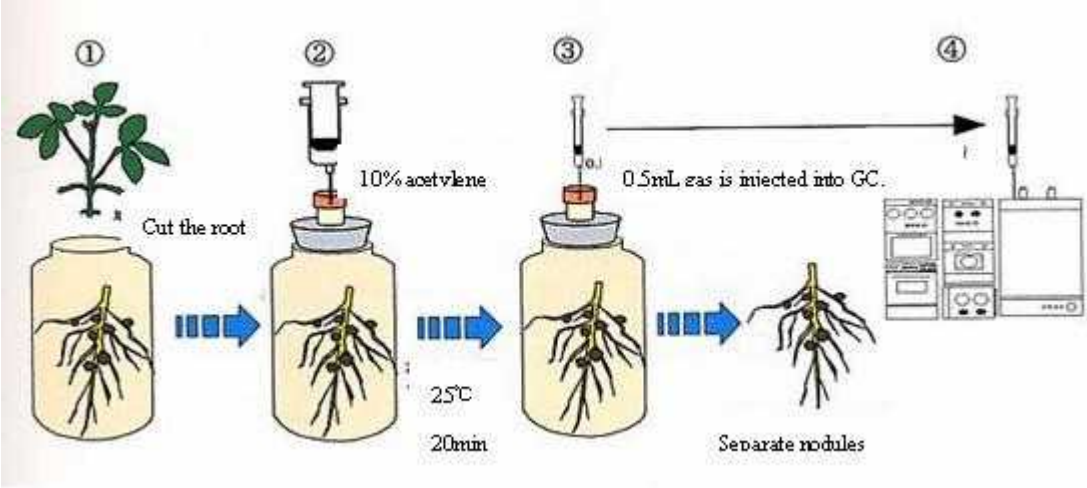


Fig.1B: ARA measurement of nodulated legumes in intact plants and detached roots

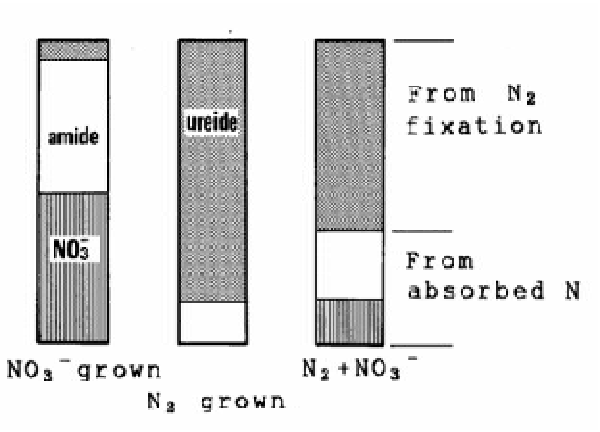
2.1.2. H_2 production assay

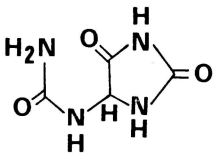
When air is replaced by inert gas such as Ar, nitrogenase catalyzes the evolution of H_2 gas instead of N_2 fixation. This H_2 evolution can be used for the index of nitrogen fixation activity like ARA (Witty and Minchin 1998). This method is used for physiological researches instead of ARA, but not applied to the field evaluation so far.

2.1.3. Relative ureide method

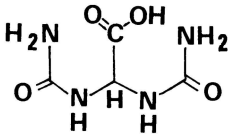
Kushizaki et al. (1964) discovered that nodulated soybean plants accumulate ureide compounds, allantoin and allantoic acids in the shoots. Comparing $^{15}N_2$ fixed in nodules and $^{15}NO_3^-$ absorbed in the roots, it was confirmed that most of the ureides in the shoots derived from nodules (Ohyama and Kumazawa 1979). Similar $^{15}N_2$ fixation studies revealed that ammonia a primary N_2 fixation product in bacteroids of a symbiotic form of rhizobia in nodules is rapidly excreted to the plant cytosol and assimilated into glutamine via the GS/GOGAT system, and synthesized to ureide via *de novo* synthesis of purine base (Ohyama and Kumazawa 1978, 1980a, 1980b, 1980c, 1981a, 1981b).

Many tropical grain legumes, such as soybean, common bean, cowpea, pigeon pea, and mung bean that have spherical determinate type of nodules transport the bulk of fixed N as ureide (allantoin and allantoic acid). On the other hand, nitrate and amino acids (especially amide, asparagine) are the major forms of N derived from soil and fertilizer N transported by roots (Ohyama and Kumazawa 1979, Ohtake et al. 1995).



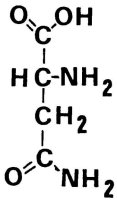


Allantoin



Allantoic acid

Nitrate



Asparagine

Fig. 2: Concept of relative ureide method and chemical structures of major nitrogen compounds in soybean xylem sap

Dr. Herridge (1990) developed the relative ureide method for evaluation of % Ndfa by analyzing the nitrogen composition of xylem sap obtained from bleeding sap from a cut stump, or vacuum collection from shoot (Fig.2, 3, 4). The concentration of ureide-N, nitrate-N and amino-N can be easily determined by colorimetry.

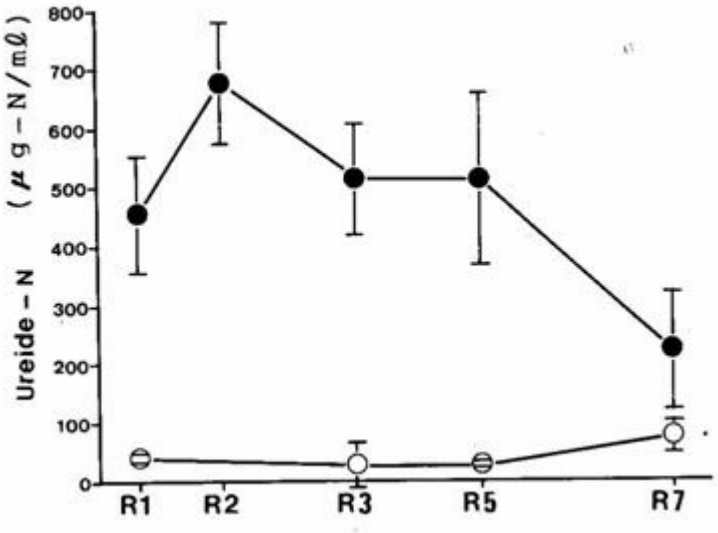


Fig. 3: Ureide-N concentrations in xylem sap of nodulating (T202) and non-nodulating (T201) isolines of soybeans

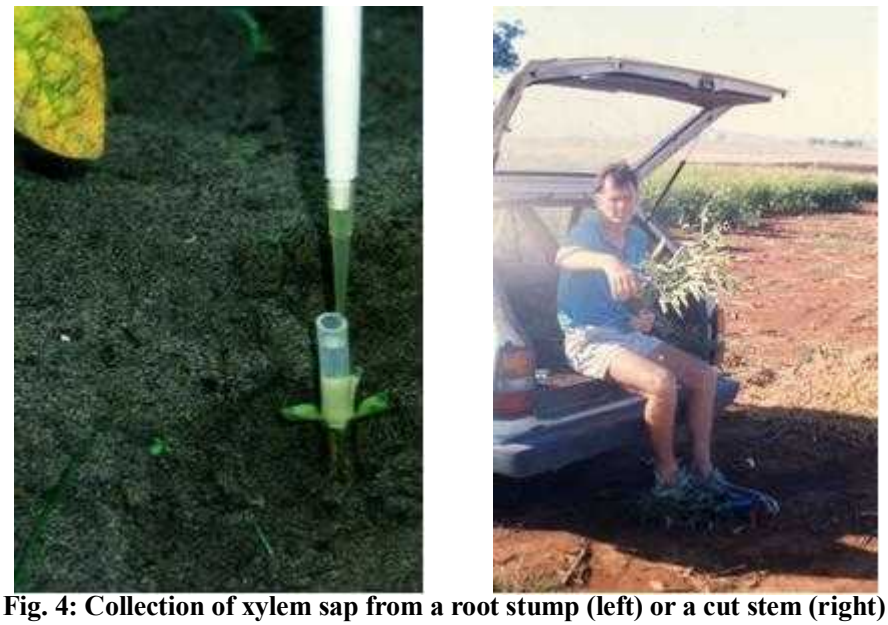


Fig. 4: Collection of xylem sap from a root stump (left) or a cut stem (right)

A study on a range of food legume species (soybean, green gram, black gram, cowpea, pigeon pea and common bean) indicated that the differences in the relationship between ureide-N and N_2 fixation are likely to be minor (Peoples and Herridge 1990).

This method is reliable in the field experiment of soybean, without any requirement of reference plants. It is the easiest way to measure %Ndfa in farmers field, because no preparation is necessary before sampling. This method is also applicable for experiments with variable N fertilizer application. In field conditions, the simple equation can be adapted for the estimation of % Ndfa.

$$\%\ \text{Ndfa} = \frac{\text{Ureide-N}}{\text{Ureide-N} + \text{Nitrate-N} + 2 \times \alpha\text{-Amino-N}} \times 100$$

The original equation proposed by Herridge was " α -Amino-N" instead of "2 x α-Amino-N" in the above equation. Based on the analysis of root bleeding xylem sap amino acids compositions, 2N amide Asparagine was the major amino acid thoughout the stages and the average N number in amino acids was 1.7, so we use "2 x α -amino-N" for the estimation.

By periodical sampling of legume plants and xylem sap, quantitative estimation of the seasonal changes in N_2 fixation activity and N absorption rate is possible（Takahashi et al. 1993). We usually sample soybean plants four or five times, at R1 (initial flowering), R3 (maximum shoot growth), R5 (pod filling) and R7 (yellow leaf) stages for xylem sap and plant N analyses.

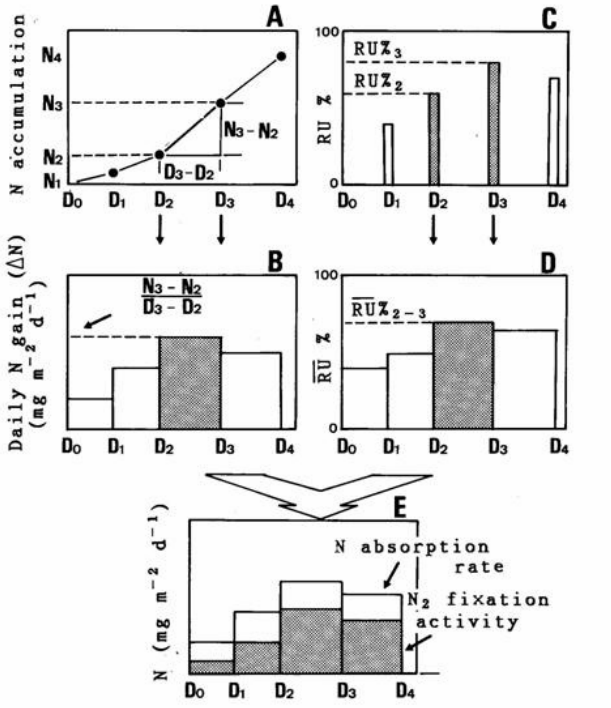


Fig. 5 shows the outline of the estimation of N_2 fixation activity and N absorption rate based on the data obtained by relative ureide method and total N analysis (Takahashi et al. 1993).

Soybean plants were harvested at D_1, D_2, D_3 and D_4. We usually harvest at R1, R3, R5 and R7 stages for D_1, D_2, D_3 and D_4. D_0 means the day of sowing. The N content of soybean shoot or whole plant including roots is determined by Kjeldahl digestion method. Average daily N increase in plant is calculated between successive sampling.

Daily N gain $\Delta N_{2\text{-}3} = (N_3 - N_2)/(D_3 - D_2)$

Fig. 5: Outline of the estimation of N_2 fixation activity and N absorption rate based on the data obtained by relative ureide method and total N analysis

The root bleeding xylem sap is collected from a cut stump for 30 min, and the percentage of relative

ureide N value (RU %) is calculated at each sampling time. The average RU % is calculated as follows:

$RU\%_{2\text{-}3} = (RU\%_2 + RU\%_3)/2$

Exceptionally, the first phase between D_0 and D_1, $RU\%_{0\text{-}1} = RU\%_1$

Combining the data of daily N gain and average RU %, the daily N_2 fixation activity and daily N absorption rate can be calculated as follows:

Daily N_2 fixation activity = $\Delta N_{2\text{-}3} \times RU\%_{2\text{-}3} /100/D_{2\text{-}3}$

Daily N absorption rate = $\Delta N_{2\text{-}3} \times (100 - RU\%_{2\text{-}3})/100/D_{2\text{-}3}$

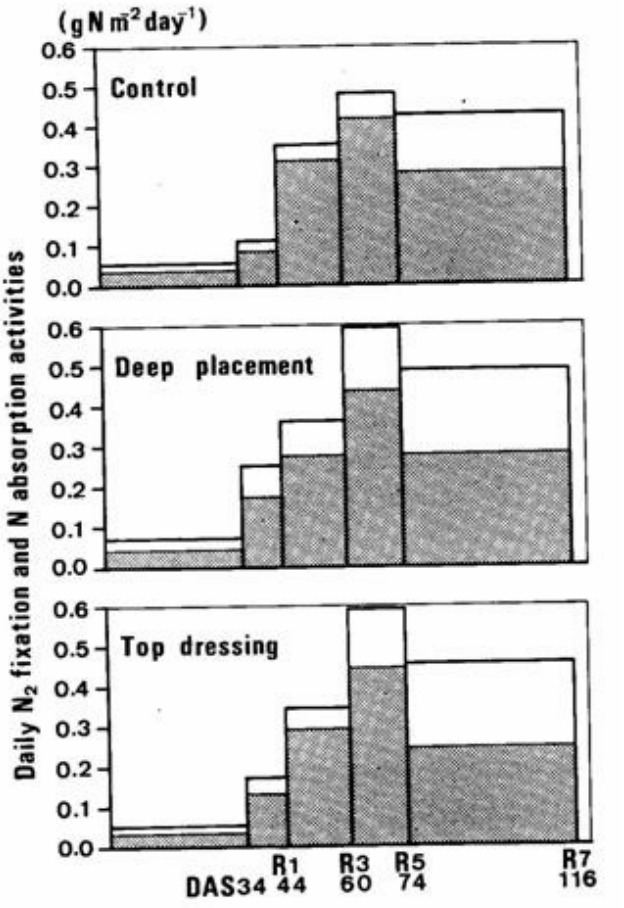


Fig. 6: Seasonal changes in daily N_2 fixation activityand N absorption rate estimated by relative ureide method (Takahashi et al.1992)

The examples of the evaluation of Ndfa by relative ureide method are shown in Fig. 6 (Takahashi et al. 1992) and Fig. 7 (Suganuma et al. 2001). A high dose of N fertilizer application generally depresses nodulation and N_2 fixation, and plant growth and seed yield are not improved in soybean cultivation. However, a deep placement of coated urea (slow release N fertilizer) did not depress N fixation and supplement N from the lower roots; plant growth and the seed yield exceeded (5.9 t/ha) the control cultivation (4.8 t/ha) (Takahashi et al 1992, 1999).

Fig. 7 shows the rate of N_2 fixation and N absorption by Williams and the hypernodulation mutants NOD1-3 that is partially tolerant to nitrate. The % Ndfa was higher in NOD1-3 (65 %) than Williams (58 %), the rate of N_2 fixation and N absorption was much lower in NOD1-3 than in Williams.

Recently, we developed the analysis of ureide-N, nitrate-N and asparagine by capillary electrophoresis (Figs. 8, 9 Sato et al. 1998).

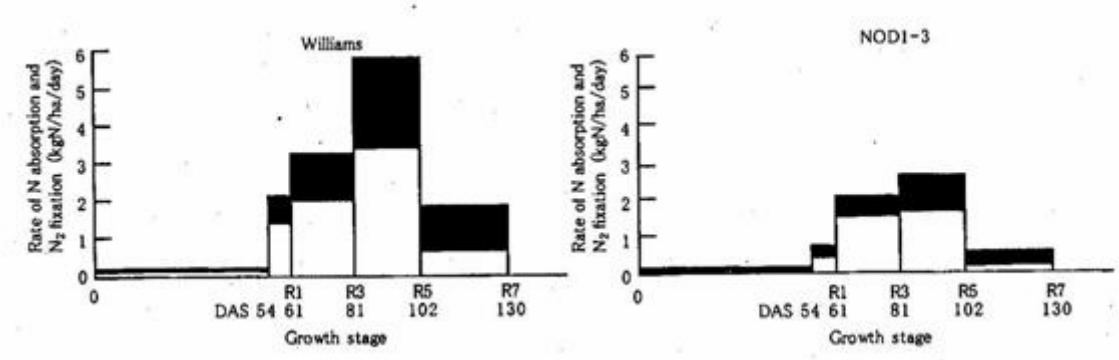


Fig. 7: Seasonal changes in daily N_2 fixation activity and N absorption rate in Willams and its hypernodulation mutant NOD1-3 cultivated in the field estimated by relative ureide method

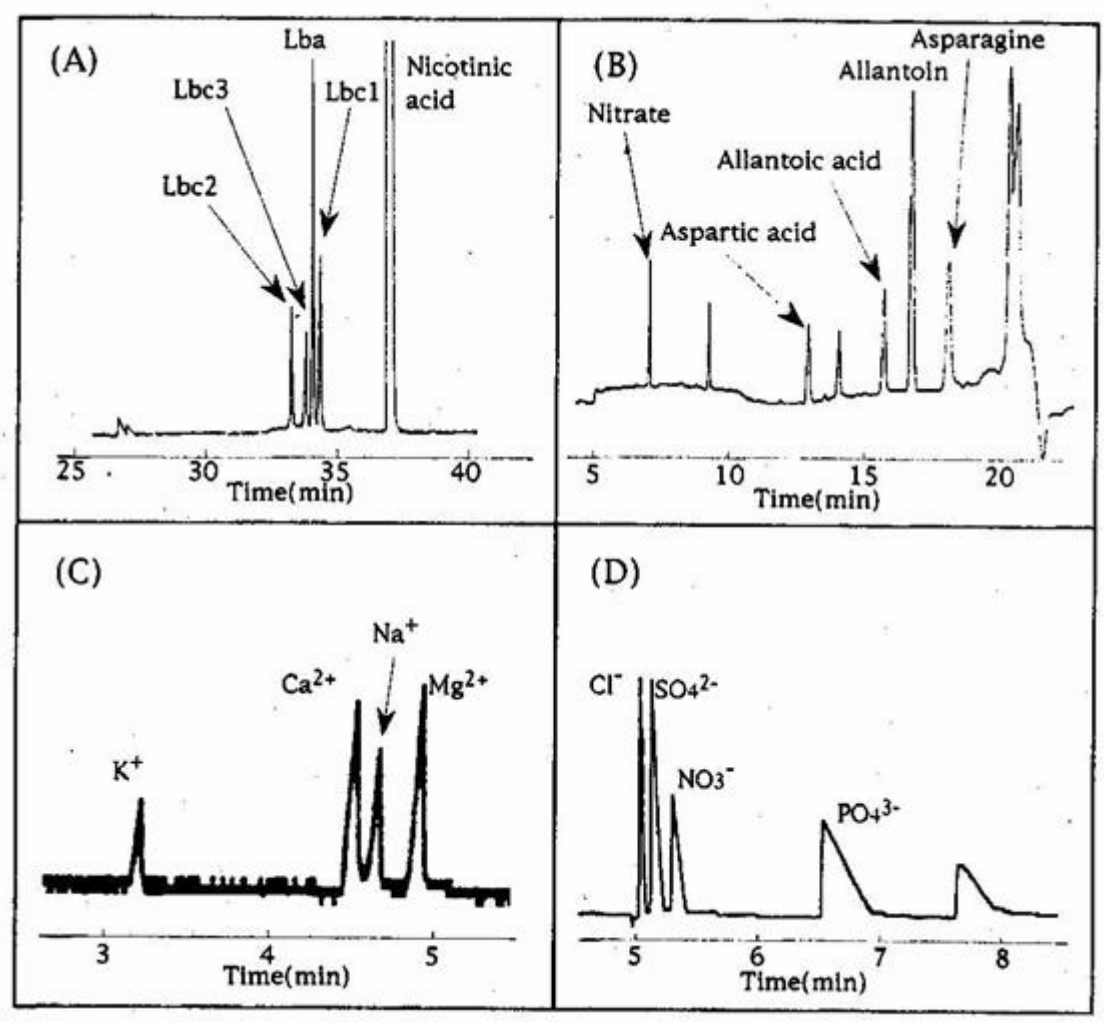


Fig. 8: Analysis of xylem solutes including N constituents by capillary electorophoresis

Fig. 9: Apparatus of Capillary Electrophoresis

Procedures for colorimetric analysis of ureide-N, amino-N and nitrate-N

The procedure below is based on the laboratory methods used in the laboratory of Prof. Dr. Harper (Univerisity of Illinois) and introduced by Takahashi and Ohyama (1993).

Ureide-N

Ureide-N concentration is determined by Young-Conway method (1942).

1) 50 μL of xylem sap is taken into a test tube
2) 3 mL of 0.083M NaOH solution is added.
3) Heat the test tubes in boiling water bath for 8 min.
   All the allantoin is degraded into allantoic acid by alkali hydrolysis.
4) Cool the test tubes in cool-water.
5) 1 mL of ice-cooled phenylhydrazinium solution is added.
   Phenylhydrazinium solution: Dissolve 0.33 g of phenylhydrazinium chloride in 100 mL of water, and add 100 mL of 0.65M HCl solution. Cool in ice water bath before use.
6) Heat the test tubes in boiling water bath for 2 min.
   All the allantoic acid is degraded into urea and glyoxilic acid.
7) Tubes are immediately cooled in ice-water bath for 15 min.
8) Add 2.5 mL of ferricyanide solution and wait for 30 min in ice-water bath.
   Ferricyanide solution: Dissolve 1.67 g of potassium ferricyanide in 100 mL of water. Add 400 mL of 10 M HCl solution. Cool in ice-water before use.
9) Measure the absorbance at 520 nm by spectrometry.

Standard 5 mM allantoin solution (280 μgN mL): Dissolve 197.7mg of allantoin in 250 mL of water.

Amino-N

α-Amino-N concentration is determined by the ninhydrin method (Herridge 1984).

1) 50 μL of xylem sap is taken into a test tube.
2) Add 1.5 mL of citrate buffer into the tube.
   Citrate buffer: Dissolve 56 g of citrate and 21.3 g of NaOH in 1 L of water.
3) Add 1.2 mL of ninhydrin solution.
   Ninhydrin solution: Dissolve 0.958 g of ninhydrin and 33.4 mg of ascorbic acid in 3.2 mL of water. Then solution is mixed with methoxyethanol (methylcellosolve) up to total of 100mL.
4) Boil the test tube in boiling water bath for 20 min with aluminum foil lid.
5) Add 3 mL of 60 % ethanol (60mL of ethanol plus 40 ml of water), and cool until room temperature.
6) Measure the absorbance at 570 nm by spectrometry.

Standard 10 mM amino acid solution (140 μg α-amino-N mL^{-1}): Dissolve 132 mg of asparagine (150mg of asparagine monohydrate) plus 146 mg glutamine in 200 mL of water. This solution contains 5mM asparagine

plus 5 mM glutamine. Total N concentration is 280 μg N mL^{-1}. Be careful to use α-amino-N concentration for the calculation of simple relative ureide equation with "2 x α-amino-N".

Nitrate-N

Nitrate-N concentration is measured by Cataldo's method (Cataldo et al. 1974).

1) 50 μL of xylem sap is taken into a test tube.
2) Add 200 μL of salicylic acid-sulfate solution into the tube, mix well and wait for 20 min.
   Salicylic acid-sulfate solution: Dissolve 5 g of salicylic acid in 100 mL of concentrated sulfric acid.
3) Add 5 mL of 2M NaOH solution into tube. Mix well and wait for 20 min.
4) Measure the absorbance at 410 nm.
   Standard nitrate solution (70 μgN mL^{-1}) : Dissolve 425 mg of sodium nitrate in 1 L of water.

2.2. P- solubilizing Activity

To determine the phosphate solubilizing activity of microorganism, the Pikovskaya's medium without agar is prepared. (see chapter 4.1.2. Isolation of mineral phosphate solubilizer)

If solubilization of rock phosphate or other insoluble phosphate is to be studied, $Ca_3 (PO_4)_2$ can be replaced by rock phosphate or other substrates containing phosphorus. The medium is sterilized in the autoclave and then inoculated with P-solubilizing microorganism. The microbe is allowed to grow in the appropiate conditions for 3 to 7 days. The clear solution is then collected by the filteration or centrifugation. The water soluble phosphorus in the clear solution is than determinated by spectrophotometric method.

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